Studies on Sertoli cell function using in vivo and in vitro models

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Declaration

The studies undertaken in this thesis were the unaided work of the author, except where acknowledgment is made by reference. The work described in this thesis has not been previously accepted for, or is currently being submitted for another degree or qualification.

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Abstract

The adult testis consists of two compartments, the seminiferous tubules and interstitium; the seminiferous tubules contain Sertoli cells (SC) each in close association with a complement of germ cells (GC) and are the site of GC maturation from diploid spermatogonia to haploid spermatozoa. The interstitium contains Leydig cells (LC), peritubular myoid cells (PTM), macrophages and blood vessels. In addition to producing haploid GC, the other essential function of the testis is synthesises of steroids hormones (primarily androgens and oestrogens). Sertoli cells are essential for support of GC development, but support of spermatogenesis is dependent upon expression of AR in SC, which they express in a stage-specific pattern. The SCs also express oestrogen receptor β (ERβ). The association between SC and adjacent GC and SC are mediated by junctional complexes, including gap junctions, tight junctions, ectoplasmic specialisations, and tubulobulbar complexes.

The aims of this thesis were; 1) to investigate androgen and oestrogen responses in SC using the immortalised SK11 cell line, 2) determine the effectiveness of an adenoviral vector for introduction of transgenes into SC *in vivo* and *in vitro*, and 3) investigate androgen-dependent gene and protein expression in testosterone depleted testes of EDS-treated rats.

SK11 cells had detectable expression of *ERβ* mRNA, and activated an ERE-*luciferase* reporter construct in response to a range of oestrogenic ligands. In contrast endogenous AR expression was low, but transfection with mouse AR cDNA resulted in sufficient AR expression to activate a luciferase reporter under control of the *Rhox5* proximal promoter in response to androgen treatment. However transfected SK11 cells could not stimulate endogenous *Rhox5* mRNA expression under similar conditions. Infection of SK11 cells with an adenoviral construct *in vitro* resulted in efficient transgene expression in 80-100% of cells, and was associated with excellent cell survival. *In vivo* infection in mouse testes with an adenoviral vector containing a GFP transgene,

performed by injection via the efferent ductules, resulted in SC-only transgene expression. When a dose of $4x10^8$ pfu was introduced the seminiferous tubule's architecture was severely damaged, invasion by macrophages and neutrophils occurred, plus expression of markers of hypoxia (Hif1α) and apoptosis (activated caspase-3 and positive Tunel assay staining) was detected. Infection with lower doses ($1x10^5$ – $1x10^7$ pfu) resulted in disruption to the seminiferous epithelium consisting of loss of pachytene germ cells and formation of intra-epithelial vacuoles. Formation of vacuoles may be due to interaction of adenovirus with the coxsackie/adenoviral receptor (CAR) in SC-SC junctions. Androgen-depletion in rats using a single dose of EDS to ablate the LC population that synthesis testosterone caused reduced expression of *AR*, *Rhox5*, *espin* and *β3-tubulin* mRNA and AR protein 6 days after treatment, but the expression and distribution of the junctional proteins espin, Cx43, zona occludins 1, and N-cadherin were unaffected.

From these studies we can conclude that SK11 cells lack vital factor/s required for activation of response elements by androgens in their endogenous promoter regions, which raises the possibility that association with other testicular cell types (GC or PTM) may be required for normal SC function to be maintained. Adenoviral vectors appear good for efficient introduction of transgenes into SC *in vitro* but not for use *in vivo*. Finally, testosterone depletion using the EDS-rat model revealed reduced mRNA expression of putative androgen responsive genes identified in previous array studies, and provides a valuable model for validation of other putative targets identified in other studies.

Presentations relating to this thesis

An immortalised cell line (SK11) derived from immature mouse testes express functionally active androgen and oestrogen receptors

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Regulation of junctional complexes in testes investigated using the EDS rat model.

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Impact of adenoviral infection on Sertoli cell function.

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Studies on the impact of adenoviral infection on Sertoli cell function

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Abbreviations

3βAdiol	5-alpha-andostane-3-beta, 17 beta-diol
ABP	androgen-binding protein
AF	activation function
AJ	adherens junction
AMH	anti-mullerian hormone
AR	androgen receptor
ARE	androgen response element
ARKO	androgen receptor knockout mouse
ArKO	aromatase knockout mouse
BSA	bovine serum albumin
BTB	blood-testis barrier
Cldn11	claudin11
Cldn3	claudin3
CP-2	cyclic protein-2
Cx43	connexin43
DAB	diaminobenzidene
DBD	DNA-binding domain
DES	Diethylstibestrol
DHT	Dihydrotestosterone
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
E2	17β-oestradiol
EDS	ethane dimethane sulphonate
ERE	oestrogen response element
ERα	oestrogen receptor α
ERαKO	oestrogen receptor α knockout
ERαβKO	oestrogen receptor α and β double knockout mouse
ERβ	oestrogen receptor β
ERβKO	oestrogen receptor β knockout mouse
ES	ectoplasmic specialisation
FORKO	FSH receptor knock out mouse
FSH	follicle-stimulating hormone
FSHR	follicle-stimulating hormone receptor
FSHβKO	follicle-stimulating hormone subunit β knockout mouse
GC	germ cell
GDNF	glial-cell line-derived neurotrophic factor
GFP	green fluorescent protein

GnRH	gonadotrophin-releasing hormone
GRE	glucocorticoid response element
Hpg	Hypogonadal mouse
HRE	hormone response element
HRP	horseradish peroxidase
Hsp	heat shock proteins
ITT	intra-testicular testosterone
Jam-1	junctional adhesion molecule 1
LBD	ligand-binding domain
LC	Leydig cell
LH	luteinising hormone
LuRKO	LH receptor knockout mice
MAA	methoxyacetic acid
MPO	myeloperoxidase
mRNA	messenger RNA
NAP	nectin-afadin-ponsin complex
NGS	normal goat serum
NR	nuclear receptors
NRS	normal rabbit serum
PBS	phosphate-buffered saline
pfu	plaque-forming units
PTM	peritubular myoid cells
RNA	ribonucleic acid
SC	Sertoli cell
SCARKO	Sertoli cell-specific androgen receptor knock out mouse
SEM	standard error of means
SGP-1	sulfated glycoprotein 1
SGP-2	sulfated glycoprotein 2
SMA	smooth muscle actin
T	testosterone
TBC	tubulobulbar complex
TBS	tris-buffered saline
TE	testosterone esters
Tfm	testicular feminised mouse
TJ	tight junction
Tubb3	β3-tubulin
WT-1	Wilms' tumour gene
ZO-1	zona occluddens 1

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1 Literature review

1.1 Introduction

The mammalian testis has two functions; the first is the synthesis and secretion of steroid sex hormones (most importantly androgens and oestrogens), which impact upon the reproductive tract and other organ systems around the body. The second is the regulation and support of germ cell maturation (spermatogenesis) to achieve the release of fully formed spermatozoa.

1.2 Organisation of the testis

The adult testis contains two distinct compartments the seminiferous tubules and the interstitium. Germ cell (GC) maturation takes place within the seminiferous tubules and is dependent upon functional support of the Sertoli cells (SC) whereas the interstitium contains the Leydig cells (LC) that express the steroidogenic enzymes essential for androgen biosynthesis.

The adult testis is closely associated with other structures in the urogenital tract, in particular the epididymis and vas deferens (Robaire and Hermo, 1988; Setchell et al., 1994). Spermatozoa released from the apical surface of SC into the lumen of seminiferous tubules are carried by the flow of tubule fluid to the rete testis, pass through the efferent ductules and thereafter enter the epididymis (Leblond and Clermont, 1952b). As spermatozoa transit through the epididymis from the caput to the cauda they mature and gain the capacity for fertilisation (Bedford, 1966). The distal end of the epididymis connects to the vas deferens that links the epididymis to the urethra, and is the tubule through which mature spermatozoa in seminal fluid pass at the point of ejaculation.

The adult testes are maintains at between 2°C and 8°C below core body temperature by virtue of being located in the scrotum (Ivell, 2007), the reduced temperature is maintained by dissipation of heat from scrotum and by a counter-current heat exchange

mechanism conducting heat from the incoming arterial blood to the outgoing venous supply (Glad Sorensen et al., 1991). These mechanisms are required because an elevation in testicular temperatures in patients or animals reduces germ cell survival (Ivell, 2007; Paul et al., 2008).

1.2.1 Seminiferous tubules

Seminiferous tubules consist of a single layer of SC associated with multiple types of germ cell organised into numerous layers. Initially the only GC present are gonocytes, but in mice between birth and 5 days postpartum they differentiate into spermatogonia prior to the first wave of spermatogenesis (Bellve et al., 1977). In the adult testes each SC is associated with a complement of GC at different stages of maturation (as shown in Figure 1-1). The lumen within the seminiferous tubules develops following formation of the blood testis barrier (BTB, see section 1.2.4.1), which occurs on or around day 14 in the mouse and day 16-19 in rats (Byers et al., 1991; Vitale et al., 1973).

In rodents the GC complement associated with each SC is the same around the full circumference of the seminiferous tubule, and the complement changes as spermatogenesis progresses. Changes in GC complement are associated with changes in SC structure and gene expression (as described in section 1.2.3). The changes in the GC-complement and structure of the epithelium follow a cyclic pattern, which is divided into the stages of spermatogenesis (see Figure 1-2). In mice the cycle is divided into 12 stages (Oakberg, 1956), whilst the rat cycle is divided into 14 (Leblond and Clermont, 1952b) and in human there are only 6 (Schulze and Rehder, 1984). In man and some primates, including the Common marmoset (Millar et al., 2000) SC within the same tubule cross-section are associated with different GC complements, in contrast to rodent tubules, the alternative arrangement has been suggested to be due either to a helical arrangement of stages within the tubule (Schulze and Rehder, 1984) or organisation of spermatogenesis into clones (Ehmcke et al., 2005). In all species each stage of the seminiferous epithelial cycle has a precise duration. Throughout successive cycles the

GC remain attached to the same SC and migrate along its surface from the basement towards the lumen from where they are eventually released, as a result the least mature diploid germ cells are positioned at the basement membrane and the most mature are found at the luminal surface (Figure 1-1, Figure 1-2). The cycles of the seminiferous epithelium, i.e. the time to progress once through stages I to VI in man or stages I to XII in mice, last approximately 16 days in man and 8.6 days in the mouse. The whole process of spermatogenesis from the time a spermatogonium becomes committed to differentiation until it is released from the seminiferous epithelium as a mature spermatozoa takes 74-76 days in man and 35 days in mice, therefore 4.6 and 4.06 cycles are required in the human and the mouse respectively (Leblond and Clermont, 1952b; Sharpe, 1994).

Tight junctions (TJ) that contribute to the blood-testis barrier (BTB) form between adjacent SC at a location between the first and second row of GC (arrow in Figure 1-1). The BTB separates the seminiferous tubule into basal and adluminal compartments, within the basal compartment spermatogonia develop up to and including the leptotene spermatocyte stage (Dym and Fawcett, 1970; Russell, 1977a). Tight junctions are not the only junctions formed within the seminiferous epithelium and gap junctions, adhering junctions (including adherens junctions (AJ), ectoplasmic specialisations (ES)), tubulobulbar complexes and desmosome-like junctions have all been described. The structure and function of these cell-cell interactions are described in more detail in section 1.2.4.

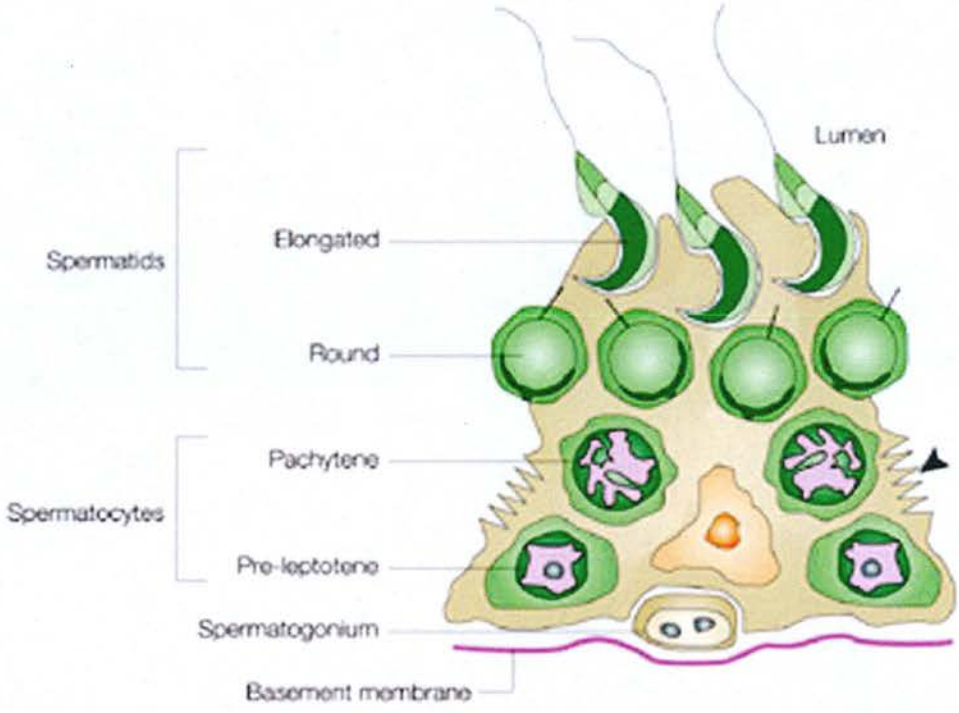


Figure 1-1: Schematic diagram of a Sertoli cell and associated germ cells. Figure shows a single SC with germ cells at various stages of spermatogenesis as indicated. Arrowhead indicates the location of the tight junctions (taken from Cooke and Saunders, 2002).

1.2.1.1 Sertoli cells

The number of SC present in adult testes is fixed; proliferation of the SC population occurs in fetal, neonatal and prepubertal periods and ceases at puberty (Sharpe et al., 2003). The number of GC associated with each SC depends on its capacity to support their development and is species-specific. Proliferation of SC in the prepubertal period is stimulated by FSH, so in mice lacking a functional FSH receptor there are fewer SC (Allan et al., 2004; Johnston et al., 2004). Androgens have also been reported to influence SC numbers in studies on mice (Johnston et al., 2004). Once SC have full germ cell complement they become functionally mature, they no longer proliferate, they change gene expression and the shape of their nuclei, and form junctional complexes

between adjacent SC and GC (Sharpe et al., 2003). The shape of SC nuclei change from round and regular in immature cells to triangular with tripartite nucleoli in adults.

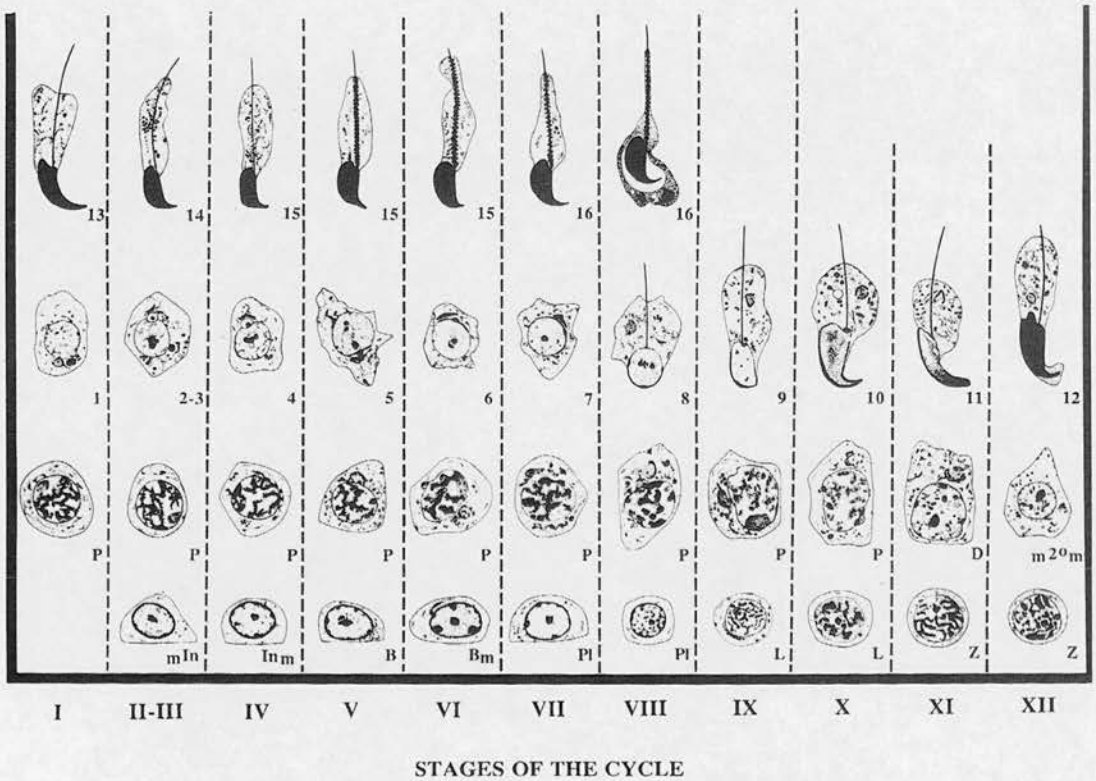


Figure 1-2: Stages of spermatogenesis in mouse seminiferous epithelium. In the figure stages of the seminiferous epithelium are presented as roman numerals, and the phases of GC development associated with each stage are presented above. Spermatogenesis progresses from left to right, and proceeds from stage XII to stage I in the row immediately above. $_{m}In - B_{m}$ are spermatogonia, Pl – $_{m}2^{o}{}_{m}$ are spermatocytes, and 1 - 16 are the stages of spermatid development (taken from summary in Russell et al., 1990).

Both maturational state and GC complement influence the proteins expressed by SC (see (Sharpe et al., 2003)), expression of anti-mullerian hormone (AMH) and aromatase decline with differentiation of SC, however Wilms' tumour gene (WT-1) expression is maintained in SC during fetal and adult life (Beau et al., 2000; Li et al., 1998; Munsterberg and Lovell-Badge, 1991; Orth and Jester, 1995; Palmero et al., 1995;

Turner et al., 2002). Indeed continued expression of AMH in adulthood signals failure of SC to mature (Sharpe et al., 2003). Expression of GATA-4 is associated with fetal and adult SC but only mature adult SC express GATA-1, which along with $p27^{kip1}$ expression are markers of initiation of spermatogenesis and cessation of proliferation (Beumer et al., 1999; Ketola et al., 2002; Yomogida et al., 1994).

SC function in the adult testis is influenced by GC complement. Features altered by GC complement include the shape of the nucleus and the pattern of gene expression (see section 1.2.3). For example, in rats at stages IX to XIV 90% of the nuclei are stretched parallel to the basement membrane (parallel-type), the remaining nuclei are stretched towards the lumen (perpendicular-type). Between stages I and VIII the number of parallel nuclei declines to approximately 60%, and perpendicular nuclei rise to 40%. The switch in nuclear orientation between stages VIII and IX is very rapid and is associated with release of mature elongated spermatids into the tubule lumen (Leblond and Clermont, 1952a).

1.2.1.2 Germ cells

The somatic cells of the fetal testis develop from the coelomic epithelium and mesonephros (Tilmann and Capel, 2002), whilst the germ cells differentiate from cell clusters within the extra-embryonic proximal epiblast (Saitou et al., 2002). The coelomic epithelium gives rise to SC and interstitial cells (Brennan and Capel, 2004; Karl and Capel, 1998) and the mesponephros contributes interstitial somatic cells that become part of the peritubular myoid cell and Leydig cell populations (Buehr et al., 1993; Merchant-Larios and Moreno-Mendoza, 1998; Tilmann and Capel, 2002). Primordial germ cells (PGC) proliferate and migrate to the gonad via the embryonal endoderm, where they associate with SC precursors and become enclosed within seminiferous cords. Once within the gonad they are known as gonocytes and in mice they undergo two periods of proliferation, the first in fetal life and the second in the early neonatal period resulting in formation of self-renewing spermatogonial stem cells

(A_s) (Clermont and Perey, 1957; Dettin et al., 2003)). In the human, proliferation and differentiation are not synchronous so a mixture of gonocytes and spermatogonia are present during fetal life (Anderson et al., 2007; Gaskell et al., 2004).

Spermatogenesis is an important process, as without the production of spermatozoa, genetic information would not be passed on (Sharpe, 1994). The adult testis contains totipotent stem cells, which continually repopulate the testis with germ cells (Huckins, 1971; Oakberg, 1971). Spermatogenesis can be split into three phases: 1) proliferative (spermatogonial), 2) meiotic and 3) spermiogenesis.

Proliferative phase

In the mouse A stem spermatogonia (A_s) divide into both new A_s cells and A paired spermatogonia (A_{pr}), which through a series of 3-4 mitotic divisions generate a population of aligned spermatogonia (A_{al}) (see Figure 1-3). The steps in the proliferation and differentiation of A_s to A_{pr} and A_{al} spermatogonia are not restricted to specific stages of the cycle in seminiferous epithelium but occur at random (Huckins, 1971; Oakberg, 1971) in the early and late stages of the cycle (stages X-II). The population of A_{al} spermatogonia differentiate during stage VII and form A_1 spermatogonia, which undergo mitotic divisions to generate A_{2-4}, intermediate (A_{In}), and A_B spermatogonia. The five successive mitotic events required to generate A_B spermatogonia from the A_1 spermatogonia are each restricted to specific stages of spermatogenesis, during stages XI-IV in mice (reviewed in Clermont and Leblond, 1953; De Rooij, 2001; Huckins, 1971; Oakberg, 1971).

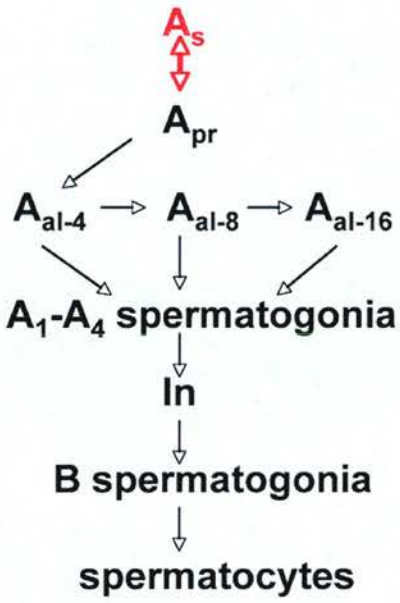


Figure 1-3: Summary of the differentiation of germ cells during the proliferative phase in the mouse. Development from a self-renewing spermatogonial stem cell (A_s, shown in red) through the stages of the proliferative phase of spermatogenesis to the point of forming pre-leptotene spermatocytes (based on De Rooij, 2001).

The proliferative phase of spermatogenesis takes place in the basal intratubular compartment of the testis. Germ cells enter the meiotic phase of spermatogenesis when A_B spermatogonia divide and become preleptotene spermatocytes during stage VI in the mouse (Russell et al., 1990; Sharpe, 1994).

Meiotic phase

The meiotic phase involves two cell divisions (meiosis I and II), during the first division the chromosome number halves, and is maintained at this haploid number following the second division (Figure 1-4). Meiosis I consists of four stages: prophase I, metaphase I, anaphase I, and telophase I. The longest stage, prophase I, can be divided further into 5 phases: leptotene, zygotene, pachytene, diplotene and diakinesis (reviewed in Cohen and Pollard, 2001). Each stage is defined by the appearance of the chromosomes in the cell, and occur at specific stages of the spermatogenic cycle. During prophase I genetic recombination occurs between homologous chromosomes at sites of synapse, which

requires a protein structure called the synaptonemal complex. Recombination at the synapse site generates additional genetic variation between the gametes formed in meiosis (reviewed in Clermont, 1972; Sharpe, 1994).

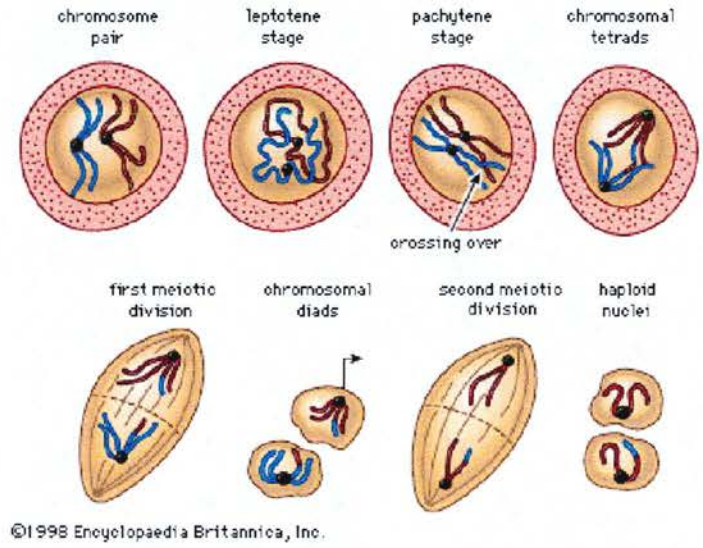


Figure 1-4: Chromosome behaviour during meiosis. Pictorial representation of the locations and structures of chromosome during Meiosis I and Meiosis II resulting in formation of haploid gametes. (Online Art. Encyclopædia Britannica Online. <http://www.britannica.com/eb/art-1688).

Spermiogenesis

The small round haploid spermatids generated at the end of meiosis II are extensively remodelled during the final phase of spermatogenesis known as spermiogenesis. Spermiogenesis can be subdivided into three phases: first development of the acrosome, second nuclear condensation and elongation and third release of the mature spermatozoon from the surface of the epithelium into the lumen. Development of the acrosome begins with formation of the pro-acrosomal vesicle by fusing together of small vesicles from the Golgi (Dooher and Bennett, 1973). The vesicle attaches to the spermatid's nuclear envelop and recruits further vesicles to enlarge its size (Russell et al., 1990; Tang et al., 1982) and spreads to cover two thirds of the nuclear surface becoming flattened in the process.

The DNA in the head of mature spermatozoon is significantly more condensed than in somatic cells, requiring six times less volume (Ward and Coffey, 1991). Condensation of the spermatozoon's DNA is achieved by changes to the DNA-binding proteins present in the cell's nucleus. The histones typical of somatic cells are replaced sequentially by testis-specific histones, and then transition proteins. Finally transition proteins are replaced by basic arginine-rich cysteine-rich proteins called protamines 1 (P1) and 2 (P2) (Balhorn et al., 1999; Oliva and Dixon, 1991). As a consequence of DNA condensation, transcriptional activity ceases early in spermiogenesis, and the germ cell must store mRNAs for proteins required later in spermiogensis in an inactive form. The mRNAs are inactivated by transcript specific RNA-binding proteins that prevent their translation for several days until they are required (Han et al., 1995; Kwon and Hecht, 1993). Elimination of water from the nucleus and cytoplasm of early elongating spermatids also contributes to nuclear condensation and reduction in cytoplasmic volume (increased density) (Sprando and Russell, 1987). The cyclic degradation and reformation of tubulobulbar complexes (described below, section 1.2.4.4) is also associated with reducing cytoplasmic and acrosomal volume (Sprando and Russell, 1987). The final process is elimination of cytoplasm and release of spermatozoa into the tubule lumen. Excess cytoplasm is released from elongated spermatids as the residual body (Leblond and Clermont, 1952b; Sprando and Russell, 1987).

1.2.2 Interstitium

The interstitium contains Leydig cells (LC), peritubular myoid cells (PTM), blood vessels and immune cells such as macrophages. Steroidogenesis is a key function of the testis and happens predominantly in the LC (see section 1.3.1). LC express all the steroidogenic enzymes necessary for biosynthesis of testosterone and oestradiol (Ge and Hardy, 1998; Turner et al., 2002). There are two populations of LC, fetal and adult (Dufau et al., 1981), the fetal population is responsible for secreting high levels of testosterone during the fetal period and is reported to be independent of LH action (El-

Gehani et al., 1998; Majdic et al., 1998). Peripheral and local hormone signals influence function of adult LC, which express LH receptors (Shan and Hardy, 1992) and AR (Bremner et al., 1994; Zhai et al., 1996). Rodent LC also contain ERα and ERß (Zhou et al., 2002) but human LC appear to lack ERα (Sierens et al., 2005).

PTM cells are located around the perimeter of the seminiferous tubules outside the basement membrane, and provide a partial barrier to diffusion of materials from the interstitium into the seminiferous tubules (Dym and Fawcett, 1970). These cells have contractile capacity and resemble the smooth muscle cells of other organs (Clermont, 1958; Ross, 1967). The location of the PTM cell layer around the periphery of the seminiferous tubules suggests a role in driving elongated spermatids released into the lumen of the tubules towards the rete testis (Clermont, 1958; Ross, 1967). In common with LC in mice they express AR, ERα and ERβ (Zhou et al., 2002). Evidence for interaction between SC and PTM cell functions have been presented by Skinner and co-workers, who report that secretion of 'P-Mod-S' by PTM cells alters SC functions including androgen-binding protein (ABP) and transferin secretion (Skinner and Fritz, 1985a; Skinner and Fritz, 1985b).

Blood vessels in the interstitium supply the testis with nutrients and signalling molecules, such as LH and FSH, carried in the bloodstream. The walls of the blood vessels do not constitute a significant barrier to the passage of substances into the testis (Dym and Fawcett, 1970). Within the interstitium there is also a resident population of macrophages, which may constitute around twenty-five percent of the interstitial cells in rats (Niemi et al., 1986). The size of macrophage population and size of the cells themselves increases as the testis matures; it has been suggested that the increased size of the cells may be consistent with the macrophage adopting a testis-specific morphology associated with an impact on steroidogenesis (Hutson, 1990). These immune cells retain their capacity to phagocytose bacterial within the interstitium (Wei et al., 1988).

1.2.3 Sertoli cell – germ cell interactions

Each Sertoli cell is physically linked to its complement of germ cells via a diverse range of junctional complexes including ectoplasmic specialisations (ES), desmosome-like junctions, gap junctions and tubulobulbar complexes (TBC) (McGinley et al., 1979; Russell, 1977a; Russell, 1977b; Russell and Clermont, 1976). Some of these junctions form alongside tight junctions, and constitute part of the blood-testis barrier, as described in section 1.2.4.1. A summary of the arrangement of the junctions is show in Figure 1-5 and the functions of each of the junctions are described in section 1.2.4.

Germ cell complement influences the pattern of gene expression in the SC, and factors secreted by SC in turn influence GC maturation. For example, the SC products activin, steel factor (kit ligand) and GDNF are reported to have an impact on spermatogonial renewal, proliferation, differentiation and adhesion (Marziali et al., 1993; Matsui et al., 1991; Meehan et al., 2000; Meng et al., 2000). Germ cell complement has a significant influence on SC function ensuring the stage-dependent expression of key SC products, examples include AR (Bremner et al., 1994), FSHR (Kliesch et al., 1992) and cyclin protein 2 (CP-2) (Maguire et al., 1993). Ablation of GC with methoxyacetic acid (MAA) in rats has revealed that expression of SC proteins including transferrin (Maguire et al., 1997), inhibin (Maddocks et al., 1992; Pineau et al., 1990) SGP-1, SGP-2 and CP-2 (McKinnell and Sharpe, 1997) ABP and AR (Tirado et al., 2003) are all influenced by a SC's meiotic and post-meiotic GC complement.

1.2.4 Junctional complexes

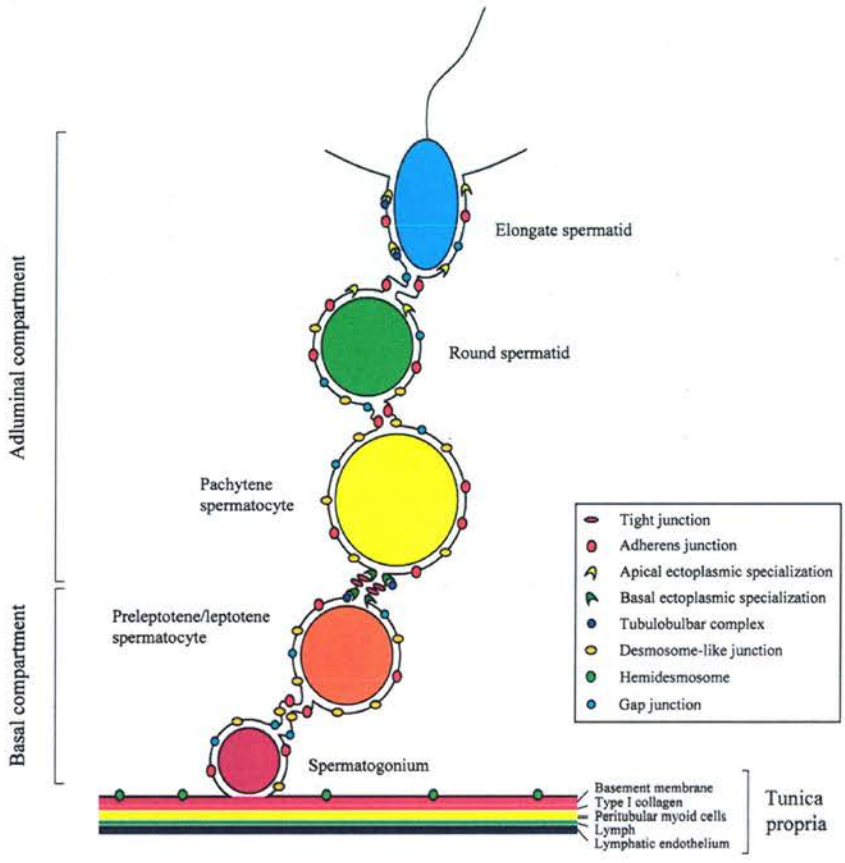


Figure 1-5: Summary of the junctional complexes described within the testis. Locations of junctional complexes between adjacent SC and adjacent SC and GC within the testis, including the multiple complexes included in the blood-testis barrier formed between adjacent SC at the border between the basal and adluminal compartment (taken from Mruk and Cheng, 2004).

1.2.4.1 Blood-testis barrier (BTB)

The blood-testis barrier is predominantly composed of tight junctions between adjacent SC found towards the base of the seminiferous epithelium that separates the tubule into the basal and adluminal compartments. Formation of the BTB around the perimeter of the adluminal compartment allows the SC to regulate composition of seminiferous tubule fluid. For example, within the adluminal compartment concentrations of androgen-binding protein, inhibin, potassium and chloride ions are higher than in

peripheral blood (Johnson and Setchell, 1968; Setchell et al., 1969; Turner et al., 1979). In contrast, the concentrations of immunoglobulins, sodium, bicarbonate, phosphate, calcium, and magnesium ions are lower (Johnson and Setchell, 1968; Setchell et al., 1969; Turner et al., 1979). The BTB also renders the adluminal compartment an immune privileged site by restricting entry of immune cells (Johnson and Setchell, 1968; Setchell et al., 1969).

Formation of the BTB is complete between postnatal days 16-19 in rats (Russell et al., 1989; Vitale et al., 1973) and around day 14 in mice (Byers et al., 1991); the formation of the BTB is associated with a dramatic increase in lumen size. Low permeability of the BTB has been demonstrated by infusion of molecular tracers such as lanthanum, horseradish peroxidase and filipin. These were excluded from the adluminal compartment and accumulated in the basal compartment when introduced into the bloodstream or the interstitium (Dym and Fawcett, 1970; Pelletier and Friend, 1983; Vitale et al., 1973).

Tight junctions in the testis consist of both integral and peripheral membrane proteins. Integral membrane tight junction proteins identified in the BTB of mice and rats include occludins (Moroi et al., 1998), claudin3 (Meng et al., 2005), claudin11 (Morita et al., 1999), Jam-1 (Yan and Cheng, 2005), and the coxsachie adenoviral receptor (CAR) (Wang et al., 2007). Associated peripheral proteins include zona occluddens 1 (ZO-1) (Byers et al., 1991; Stevenson et al., 1986) and ZO-2 (Jesaitis and Goodenough, 1994).

ZO-1 is localised to the cytoplasmic region of the tight junctions (Stevenson et al., 1986) and, in common with ZO-2 possess binding domains for both the actin cytoskeleton and associated tight junction proteins (Fanning et al., 1998). ZO-1 has the capacity to bind the following tight junction components: ZO-2 (Fanning et al., 1998), occludins (Moroi et al., 1998), claudins (Itoh et al., 1999) and Jam-1 (Bazzoni et al., 2000). ZO-1 links the tight junction complex to the actin cytoskeleton (Fanning et al., 1998). Within TJ complexes ZO-2 and occludin interact directly, binding each other and actin side-chains

(Wittchen et al., 1999) and interactions between ZO-2 and claudins are also reported (Itoh et al., 1999).

The BTB also includes adherens junctions (AJ), called basal ectoplasmic specialisations (ES), that consist of a cadherin-catenin structure (section 1.2.4.2). Expression of N-cadherin, α-catenin, β-catenin, γ-catenin and p120ctn (Byers et al., 1994; Johnson and Boekelheide, 2002a; Johnson and Boekelheide, 2002b), which are key constituents of the cadherin-catenin complexes, have all been detected at basal ES of the BTB.

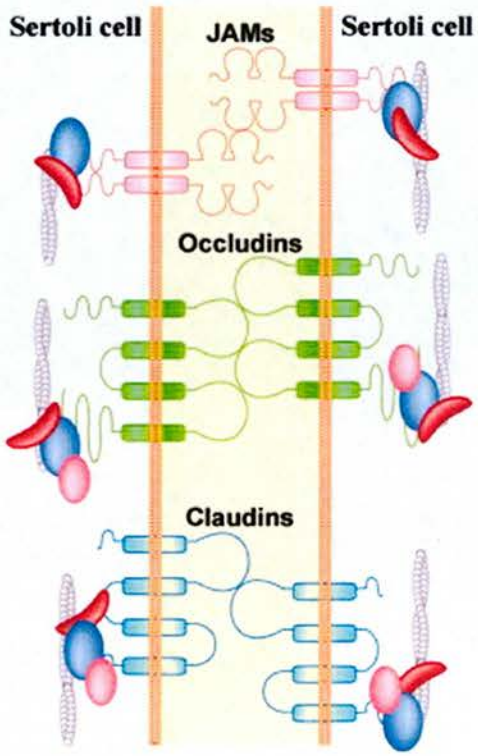


Figure 1-6: Tight junction complexes between adjacent Sertoli cells. Three tight junction complex adaptor proteins associated with ZO-1 (blue subunit), ZO-2 (pink subunit), and afadin (red subunit) present between adjacent SC within the testis (taken from Lee and Cheng, 2004)

Claudins interact with ZO-1, ZO-2, ZO-3 (ZO-3, has not been shown to be expressed in testis). Claudin11 is highly expressed in brain, testis, and can be found in the kidney. In

the testis claudin11 is an important constituent of TJ between SC. (Lui et al., 2003). The BTB must allow passage of GC from the basal to adluminal compartment of the seminiferous tubule without diminishing its barrier function and this may be achieved by dissociation between TJ and AJ adaptors, and dissociation of AJ. The stability and barrier function of the BTB is maintained during these dynamics changes by increased TJ expression (Yan and Cheng, 2005).

1.2.4.2 Ectoplasmic specialisation

Ectoplasmic specialisations (ES) are specialised forms of AJ found uniquely in the testes. They consist of cistern of endoplasmic reticulum and the SC plasma membrane on alternate sides of actin filaments bundles, linked through complex protein associations. The structure is symmetrical in SC-SC junctions, but asymmetrical in SC-GC with microfilaments bundles only in SC (Grove and Vogl, 1989). The ES within the seminiferous tubules are separated into two groups based upon the compartment in which they are expressed: basal or adluminal. The ES junctions at each site consist of at least one of the following three distinct complexes of proteins: a cadherin-catenin complex, a nectin-afadin-ponsin (NAP) complex or an integrin-laminin complex (Chapin et al., 2001; Mruk and Cheng, 2004; Ozaki-Kuroda et al., 2002; Siu and Cheng, 2004; Wine and Chapin, 1999).

Basal ES form part of the BTB between adjacent SC; the junctions formed at this site consist of cadherin-catenin complexes that interact with the underlying actin cytoskeleton. The cadherin-catenin complex involves cadherins interacting with β-catenin/γ-catenin, which initiates interaction of the N-terminal of β- or γ-catenin with α-catenin. The α-catenin C-terminal interacts with actin either directly or via actin-binding molecules (α-actinin, vinculin, or zyxin) (Grove and Vogl, 1989; Lee et al., 2004; Li and Trueb, 2001; Mruk and Cheng, 2004; Vogl et al., 2000)

Two complexes are believed to exist at the apical ES; these are the NAP and integrin-laminin complexes (Siu and Cheng, 2004). The presence of cadherin-catenin complexes at this site is controversial, with conflicting reports on the expression of N-cadherin, β-catenin and p120ctn. The nectin/afadin/ponsin complex, consists of nectin in association with L-afadin. Nectin-3 is expressed strongly in spermatids, nectin-2 is expressed in SC. They are expressed at junctions between SC and spermatids in a pattern typical of proteins in the apical ES (Ozaki-Kuroda et al., 2002).

ES in the adluminal compartment are associated with GC from the mature pachytene spermatocytes stage (late in stage VII rat epithelium) up to and including late spermatids (stage 19, stage VII in rats) (Russell, 1977b). The ES associated with spermatids at stage 7 or later are believed to retain GC association with SC by maintaining recesses in which spermatid heads are held. Recesses are lost at stage VII and loss is associated with altered ES position (Russell, 1977b). ES are associated with 1) GC as their position changes within the seminiferous epithelium, and 2) microtubules, which suggests SC controls GC movement via ES and microtubules (Russell, 1977b).

The ES closely associated with the BTB are basal ES, they consist of actin filaments associated with endoplasmic reticulum close to the internal surface of the plasma membrane and interacting proteins including ZO-1, vinculin, N-cadherin, β1-integrin, integrin linked kinase (ILK) and espin (Bartles et al., 1996; Dym and Fawcett, 1970; Grove and Vogl, 1989; Mulholland et al., 2001; Pfeiffer and Vogl, 1991). Identification of α6β1 integrin in a basal location associated with BTB implicates the integrin-laminin complex in basal ES, this complex breaks down when the preleptotene spermatocytes cross the BTB (Salanova et al., 1995).

Espin is a testis specific protein expressed exclusively by SC in the seminiferous epithelium, it is localised to both basal and adluminal ES between adjacent SC and SC and GC, respectively. In the adluminal compartment, espin is localised between SC and the heads of elongate spermatids from mid to late spermiogenesis. The expression of

espin follows the typical pattern of ES formation and then breakdown seen during spermiogenesis. Basal expression of espin is seen between SC in a pattern characteristic of basal ES. Espin was shown to be capable of binding F-actin (Bartles et al., 1996).

ZO-1 is localised to ES with round and elongating spermatids but expression is lost prior to spermiation (Byers et al., 1991). CAR is co-localised with espin at apical ES between SC and elongating spermatids in stage VIII rat tubules (Wang et al., 2007). Fimbrin and vinculin are strongly associated with ES enriched fractions in rat testes, and vinculin is immunolocalised to SC-SC and SC-spermatid junctions. Fimbrin is an actin-binding protein.

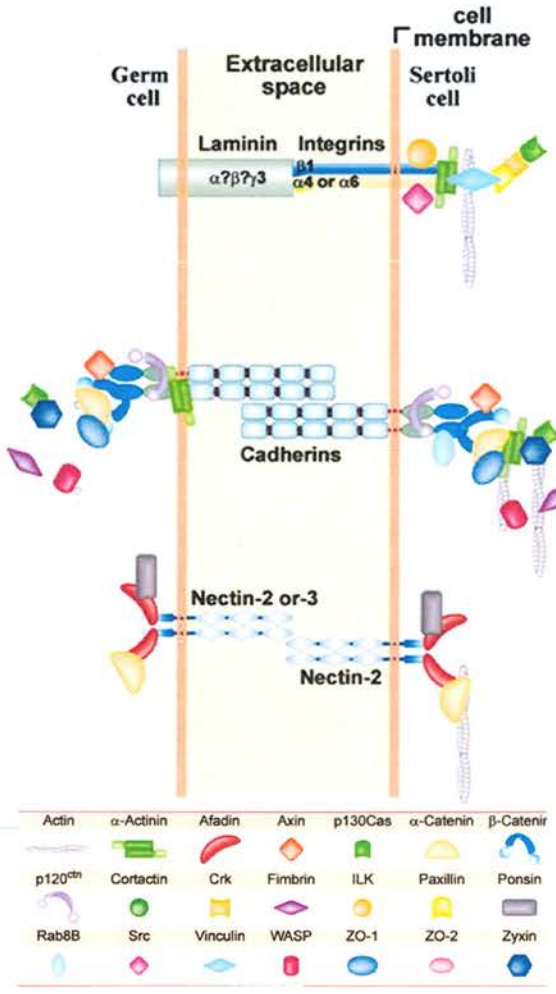


Figure 1-7: Ectoplasmic specialisation complexes between Sertoli cells and germ cells. The three adherens junction complexes present in ES within the testis: integrin- laminin, cadherin-catenin, nectin-afadin-ponsin plus associated proteins. The figure also indicates the components contributed by the SC and GC at SC-GC ectoplasmic specialisations (taken from Lee and Cheng, 2004)

1.2.4.3 Gap junctions

Gap junctions are channels that connect the cytoplasm of adjacent cells; each channel is composed of two subunits (connexons) made up of six connexin proteins. Gap junctions allow the passage of small molecules and ions (Kumar and Gilula, 1996; Musil and

Goodenough, 1993). At least twenty connexins have been identified, of which eleven have been identified in testes (Pointis et al., 2005; Risley, 2000). Of the eleven connexins in testes, eight are expressed in SC and nine in GCs. The most abundant of these, connexin43 (Cx43), is expressed in SC, GC and LC (Risley et al., 1992; Roscoe et al., 2001). In adult testes expression of Cx43 is found between adjacent SC and between SC and GC, from spermatogonia up to and including pachytene spermatocytes (Batias et al., 1999; Batias et al., 2000). The SC-SC gap junctions co-localise with ZO-1 and are associated with the BTB (Batias et al., 1999; Giepmans and Moolenaar, 1998). In rats expression of Cx43 within the SC is stage-specific, expression is weak during stages V-VI, reaching a peak in stage VII and declining again at stage IX of spermatogenesis (Batias et al., 1999; Batias et al., 2000; Risley et al., 1992). The importance of Cx43 has been confirmed using targeted gene deletion in mice. Connexin 43 null mutant mice have a germ cell deficiency that develops during fetal development and persists into the early neonatal period during which the mutant mouse dies (Juneja et al., 1999). If neonatal development of the testis is extended in grafting experiments the germ cell deficiency is maintained resulting in a postnatal "Sertoli cell only" phenotype (Roscoe et al., 2001). When Cx43 expression is knocked out specifically in SC, studies reveal spermatogenesis arrests at the spermatogonial stage, and can result in SC-only syndrome with clusters of intratubular SC are observed in the testes (Brehm et al., 2007).

1.2.4.4 Tubulobulbar complexes

The tubulobulbar complex is a structure that originates from the acrosomal membrane of elongated spermatids and projects into the cytoplasm of associated SC. The TBC consists of tubules with a bulbous mid-section that interact with the SC via bristle-coated pits (Russell and Clermont, 1976; Russell and Malone, 1980). In rats, and the majority of other species, the TBC is maintained until the point of spermiation, but in mice the TBC cannot be identified (Russell and Malone, 1980). The TBC occupy the same sites as the apical ES between SC and elongating spermatids and have been suggested to play a role in the removal of apical ES by internalisation of the junctions, and to replace the

ES in maintaining spermatids at the luminal surface of SC until they are released to the lumen (Guttman et al., 2004; Russell and Malone, 1980). There is also evidence that cycles of TBC formation and phagocytosis by the SC result in elimination of cytoplasm from the acrosome of the spermatids (Russell and Malone, 1980; Tanii et al., 1999). TBC are also present at basal locations between adjacent SC in the seminiferous tubules where they may play a role in gap junction elimination indicated by loss of Cx43 expression (Risley et al., 1992; Russell and Clermont, 1976; Tanii et al., 1999).

1.2.4.5 Desmosome-like junctions

Desmosome-like junctions are involved in adherence of spermatogonia, spermatocytes and round spermatids to the SC in the seminiferous epithelium and in the transfer of GC from the basal to adluminal compartment of the seminiferous tubules (Russell, 1977a).

These are actin-associated anchoring-junctions. During migration of spermatocytes across the BTB barrier into the adluminal compartment, desmosome-like structures develop between the SC and pre-leptotene spermatocytes. These junctions attach the spermatocytes to the SC resulting in the cell being drawn away from the basal membrane and ultimately crossing the BTB (Russell, 1977a). Desmosome-like junctions interact with intermediate filaments (Mulholland et al., 2001). N-cadherin is expressed in a stage independent manner in desmosome-like junctions and is co-localised with β-catenin and p120 (Johnson and Boekelheide, 2002b; Mulholland et al., 2001).

1.3 Steroids

1.3.1 Steroidogenesis

Leydig cells are the site of testosterone synthesis (Christensen and Mason, 1965; Eik-Nes and Hall, 1965; Hall et al., 1969), and oestrogen is synthesised in the following testicular cells that express the aromatase enzyme: LC, immature SC, and subpopulations of GC (Kurosumi et al., 1985; Turner et al., 2002). The key steps,

intermediate products and enzymes of the steroidogenic pathway in LC are shown in Figure 1-8.

Cholesterol is the initial substrate for the process of steroidogenesis, so must accumulate in mitocondria at the site of the first enzyme reaction (Miller, 2007). The initial transport of cholesterol into the mitochondria requires cholesterol to be mobilised from cellular stores and transported to the outer mitochondrial membrane, a further translocation of cholesterol to the inner mitochondrial membrane is required to reach the first site of cholesterol's conversion to steroids (Stocco and Clark, 1996). Initial intracellular transport of cholesterol appears to involve the StarD4, 5, and 6 proteins (Soccio and Breslow, 2003). Cholesterol transport within the mitochondria to the inner mitochondrial membrane is mediated by StAR (steroidogenic acute regulatory) protein (Clark et al., 1994; Stocco and Sodeman, 1991), and is the rate limiting step during steroidogenesis and the point at which acute regulation of steroidogenesis occurs (Crivello and Jefcoate, 1980; Miller, 2007). Synthesis of the StAR protein in LC is in response to hormone stimulation, by LH and human chorionic gonadotrophin, StAR is initially synthesised as 37 then 32 kDa precursor proteins which are processed to four forms of 30 kDa proteins that are localised to the inner mitochondrial membrane (Clark et al., 1994; Stocco and Sodeman, 1991). The mechanism for import of cholesterol to the inner mitochondrial membrane has not been determined, but it has been determined that StAR is only functional transiently at the cytoplasmic surface of the outer mitochondrial membrane and not at its final destination in the matrix (Bose et al., 2002). Investigations have identified potential interactions at the outer mitochondrial membrane between StAR and TSPO, PAP7, and PKAIα. These four units function together in mitochondrial cholesterol import (Liu et al., 2006).

Chronic regulation of steroidogenesis is via expression of the steroidogenic enzymes that catalyses the conversion of cholesterol to pregnenolone, through to androstenedione, and onwards to androgens and oestrogens; control that is mediated by LH action (Miller, 2007; Payne and Youngblood, 1995). The steps of steroidogenesis are catalysed by a

series of enzymes including cytochrome P450s, hydroxysteroid dehydrogenases (HSD) and 5α-reductase. The initial conversion of cholesterol to prenenolone is the rate limiting enzyme reaction (Karaboyas, 1965) located at the inner mitochondrial membrane and catalysed by P450scc (side-chain cleavage) on the mitochondrial matrix side of the membrane (Churchill and Kimura, 1979). The action of P450scc catalyses 3 reactions 1) 22-hydroxylation, 2) 20-hydroxylation, and 3) scission of 20-22 carbon-carbon bond (Hall, 1994)}(Miller, 2002; Simpson, 1979; Yago and Ichii, 1969):

Cholesterol → 22-OH Cholesterol → 20,22-Di-OH-Cholesterol → Pregnenolone

The synthesis of androstenedione from pregnenolone occurs in the microsomes of the mitochondria (Hall, 1994) and can be achieved through two alternative pathways (reviewed in (Conley and Bird, 1997)) dependent upon the order in which the pregnenolone undergoes a) hydroxylation and lysis by P450c17 and b) dehydrogenation by 3β-HSD type I (Bain et al., 1991). If dehydrogenation precedes P450c17 actions, steroidogenesis follows the Δ4 pathway:

Pregnenolone → Progesterone (catalysed by 3β HSD)

Progesterone → 17-OH Progesterone → Androstenedione (catalysed by P450c17)

If P450c17 acts first the Δ5 pathway is followed:

Pregnenolone → 17-OH Pregnenolone → Dehydroepiandrosterone
(catalysed by P450c17)

Dehydroepiandrosterone → Androstenedione (catalysed by 3β HSD)

Both yield the same product but there are species differences between which pathways are utilised. Rats use the Δ4 pathway almost exclusively (Hall, 1994), whilst pigs, rabbits, dogs and man use the Δ5 pathway (Hall, 1994). The importance of Δ4 versus Δ5 pathway in different species is dependent upon variations in the properties of 3β HSD and P450c17, including relative affinity of the enzymes for their substrates, relative expression levels, and efficiencies of the reactions (Conley and Bird, 1997). In the case of humans the Δ5 pathway is used because of an inability to catalyse 17-OH

Progesterone → Androstenedione in the Δ4 pathway (Conley and Bird, 1997). Androstenedione is the precursor of testosterone and the conversion is catalysed by 17β HSD type III, which is detected in the mouse testis within the microsomes (Penning, 1997; Sha et al., 1997). Testosterone generated in the testis can be converted further to form the more potent androgen DHT (Dihydrotestosterone) a conversion catalysed by 5α-reductase type I in mice (O'Shaughnessy et al., 2002). Aromatase (P450arom) is expressed in mature testes by LC and some GCs (Turner et al., 2002) (Levallet et al., 1998), this enzyme converts testosterone to oestradiol and this conversion is stimulated in LC by LH/hCG (Valladares and Payne, 1979; Valladares and Payne, 1981).

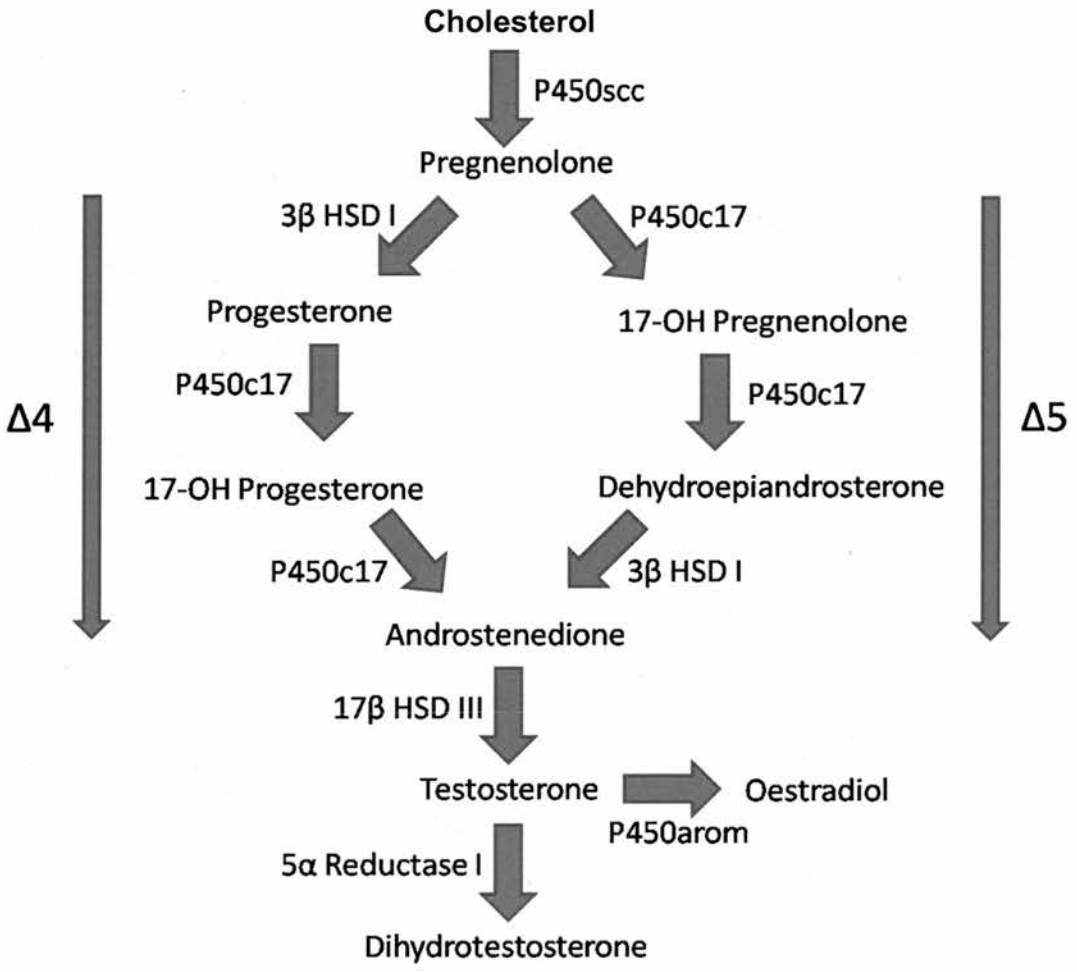


Figure 1-8: Synthesis of sex steroids. Pathway for biosynthesis of androgens and oestradiol from cholesterol, indicating the substrates, enzymes and products of each step, based upon figure in (Conley and Bird, 1997).

1.3.2 Steroid receptors

1.3.2.1 Structure and signalling

The sex steroid receptors are members of a super-family of genes that typically function as ligand-activated transcription factors (Bain et al., 2007) although some NR

superfamily members are reported to act in the absence of a cognate ligand. The members of the family have a conserved arrangement of functional domains depicted in Figure 1-9. The generalised structure of a nuclear sex steroid receptor consists of six domains, called A to F. The A/B domain contains the activation function (AF)-1 region, domain C is the highly conserved DNA-binding domain (DBD), D is the hinge domain and is proximal to the E/F domain that contains the ligand-binding (LBD) domain and AF-2 region (Olefsky, 2001). The DBD is the site of interaction between steroid receptors and their hormone response elements (HRE) within promoter regions of responsive genes, and contains two zinc-finger regions. The first zinc finger ensures that interaction between the DBD and response elements are sequence specific, whilst the second zinc finger determines the spacing between units of the response element to which the domain can bind (Bain et al., 2007; Ribeiro et al., 1995). The LBD is less conserved than the DBD but includes a binding pocket that confers ligand specificity and the second activation function (AF-2 region). (Bain et al., 2007). In the progesterone receptor mutations within the AF1 and hinge domains result in loss of transcriptional activity (Dobson et al., 1989).

Binding of ligand to sex steroid receptors causes a change in the LBD from an inactive to an active conformation (Beekman et al., 1993; Kuil et al., 1995) and dissociates the receptor from a complex of chaperone proteins including Hsp90, Hsp70 and Hsp56. Numerous proteins have been reported to interact with the region AF2, some of which have the properties of transcriptional coactivators including those shown in Figure 1-10. For example, studies by Parker and coworkers have demonstrated that recruitment of the p160 co-activators to the ER is dependent on the integrity of a C-terminal helix, referred to as helix 12 (Heery et al., 1997), which structural analysis suggests is realigned during binding of ligands to the LBD domains of several receptors (Renaud et al., 1995; Schulman et al., 1996). In the ER it is misaligned in the presence of an antiestrogen, raloxifene, which blocks AF2 activity (Brzozowski et al., 1997).

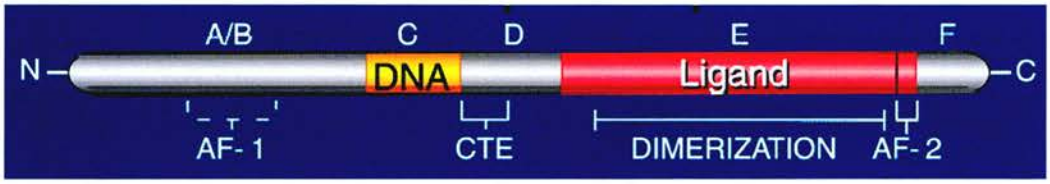


Figure 1-9: Steroid receptor structure. Schematic diagram of the generalised structure of a steroid receptor including sites of key functional domains (taken from Olefsky, 2001).

The human androgen receptor (*AR/ NR3C4*) cDNA was cloned in 1988, and localised to the X chromosome (Lubahn et al., 1988; Trapman et al., 1988); rat and mouse homologues have also been cloned (Chang et al., 1988; Faber et al., 1991). There are two oestrogen receptors known as *ERα* (*ESR1*) and *ERβ* (*ESR2*); *ERα* was cloned from human MCF-7 in 1985 (Walter et al., 1985) and from the mouse uterus in 1987 (White et al., 1987). *ERβ*, was identified a decade later in rats and humans (Kuiper et al., 1996), and the cDNA sequence in the mouse was published in 1997 (Tremblay et al., 1997). The ERα and ERβ proteins both possess DBD and LBD with significant homology (97% and 60%, respectively), but the N-termini are not homologous (Tremblay et al., 1997). *ERβ* has two splice variants in mice *ERβ1* and *ERβ2*, the β2 variant contains an additional eighteen amino acids within its LBD. The affinity and transcriptional capacity of the ERβ2 variant is significantly reduced compared to the ERβ1 form, this is proposed to be due to reduced interactions with transcription co-factors (Zhao et al., 2005). Multiple splice variants of *ERβ* have been identified in human none of which are homologous to the mouse variants (Moore et al., 1998).

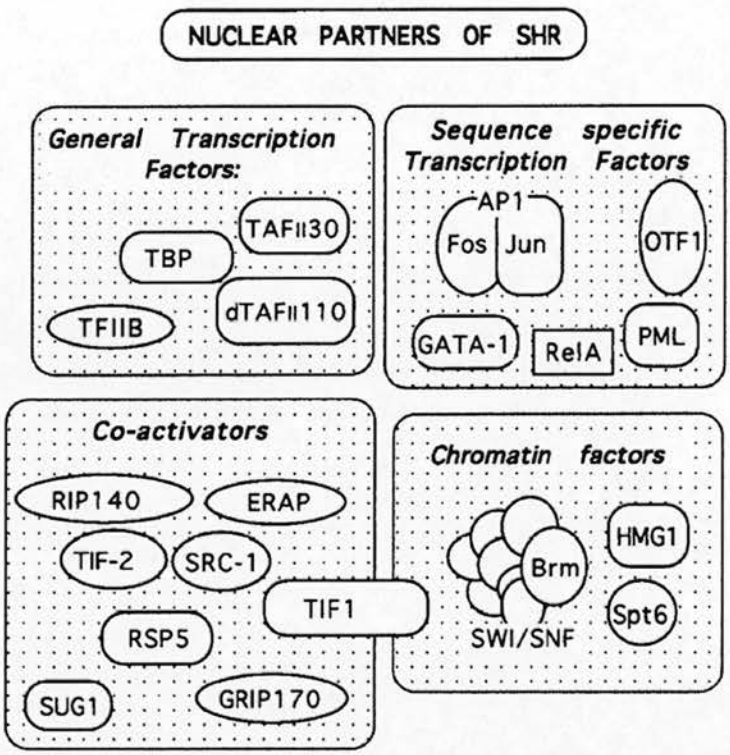


Figure 1-10: Nuclear partners of steroid hormone receptors. Co-factors associated with steroid hormone receptors required for induction of transcription by the activated steroid receptor. Factors are group by function. (taken from Beato and Sanchez-Pacheco, 1996).

The steroid receptors either function as homo- or hetero-dimers; dimerisation is dependent upon sequences within both the DBD and LBD, with the C-terminal of the LBD exerting the greatest influence (Tsai and O'Malley, 1994). Monomeric AR is unable to bind to androgen response elements (AREs) in gene promoters and can only function as a dimer. Deletion studies have shown that homo-dimerisation of AR is inhibited in the absence of bound androgens by the N-terminal domain. The binding of the ligand counteracts the inhibition by altering the conformation of the receptor resulting in DNA-binding by AR dimers (Wong et al., 1993). A classical ARE sequence was identified by Roche et al (Roche et al., 1992) and described as consisting of two half-sites (inverted repeats) separated by a 3 base-pair region. The ARE sequence (5'-GGA/TACANNNTGTTCT-3') bears significant homology to the glucocorticoid

response element (GRE) that is recognised by the glucocorticoid and mineralocorticoid receptors. The size of the spacer region between half-sites is important to maintaining the function of the ARE and an increase or decrease of just one nucleotide prevents formation of the receptor-DNA complex (Kallio et al., 1994). Binding of androgens by rat and human AR via the LBD of the receptor protein is required for intramolecular interaction between the C-terminal LBD and N-terminal transactivation domain, including the AF-1 site (Doesburg et al., 1997; Ikonen et al., 1997). The intramolecular interactions and resultant activity of the complex both require the involvement of nuclear co-factors including CBP and F-SRC-1 (Doesburg et al., 1997; Ikonen et al., 1997). CBP in particular has been identified as recruiting RNA polymerase II and transcription factor IIB (Doesburg et al., 1997).

The *Rhox5* gene expressed in the SC and epididymis contains two AREs (ARE-1 and ARE-2) that differ slightly from the consensus ARE. For example, the half-sites of ARE-1 consist of two direct repeats separated by five nucleotides and ARE-2 also consists of half-sites with a structure that approximates direct repeats separated by three nucleotides. The process of AR binding to ARE-1 and -2 within *Rhox5*'s proximal promoter is dependent upon the right half-sites, which suggested AR binding to the right half-site is the first step in a two-step ARE-binding process, the second step being binding to the other half-site which is dependent on binding to the right half-site (Barbulescu et al., 2001).

1.3.3 Impact of hormones on male reproduction

Hormones are central to the regulation of male fertility; an overview of the hormones involved in regulating adult male reproduction is shown in Figure 1-11. Regulation is dependent upon stimulation of the testis by the pituitary gonadotrophins LH and FSH and negative feedback by steroids and proteins (e.g. inhibin) produced by the testis.

1.3.3.1 Gonadotrophins

The gonadotrophins LH and FSH are synthesised and secreted by the gonadotropes within the anterior lobe of the pituitary gland. The gonadotrophins are heterodimeric hormones consisting of a common alpha protein chain and a beta chain that identifies the hormone, and determines interactions with the hormone receptor (Combarnous, 1992; Haisenleder et al., 1994). Secretion of LH and FSH from the anterior pituitary is stimulated by the hypothalamus via a ten amino acid peptide called gonadotrophin-releasing hormone (GnRH), which is secreted in regular pulses (Haisenleder et al., 1994). The LH receptor is expressed by LC, and FSH receptor by SC within testes (Kliesch et al., 1992; Shan and Hardy, 1992). The importance of FSH in determining the final number of SC within testes is demonstrated in mice in which FSH signalling is disrupted, at five days postpartum these mice have normal SC numbers but they are reduced by 40% in adult testes at 20 days postpartum (Johnston et al., 2004). The importance of gonadotrophins in male fertility has been demonstrated in models such as the *Hpg* mouse, and mice with targeted disruption of LH or FSH signalling. The hypogonadal (*Hpg*) mouse carries a mutation in the GnRH gene (Mason et al., 1986) which causes a permanent reduction of both LH and FSH levels and consequentially failure of SC and GC to mature (Myers et al., 2005). Treatment of *Hpg* mice with LH can reinstate steroidogenesis (Scott et al., 1990) and treatment with FSH plus androgens restores qualitatively normal spermatogenesis (Haywood et al., 2003; Singh and Handelsman, 1996). LH and FSH can be withdrawn in adulthood by hypophysectomy (surgical or via GnRH antagonist), animals undergoing these treatments exhibit reduced testis weight, and after three weeks reduced numbers of spermatocytes, and loss of spermatids (El Shennawy et al., 1998). Deletion of the LH receptor in mice (LuRKO mice) results in abnormal development of their LC, which are reduced in size and number, and is associated with disruption of spermatogenesis in round spermatids (Zhang et al., 2001). The testes of mice with disruption of FSH signalling due to absence of a functional FSH receptor (FORKO) or FSH beta chain (FSHβKO) contain a reduced number of SC, and retarded development with puberty and appearance of spermatocytes delayed in these animals (Johnston et al., 2004; Krishnamurthy et al.,

2001). Introduction of 1) a constitutively active FSHR transgene or 2) a transgene containing tandem alpha and beta chains of FSH, into *Hpg* mice can overcome the disrupted *Hpg* phenotype increasing testis weight and supporting development of germ cells up to the round spermatid stage, however few elongated spermatids are generated (Allan et al., 2004).

Androgens and oestrogens produced by the testis are important for normal spermatogenesis (see evidence below) and together with inhibin also provide negative feedback on the anterior pituitary to regulate gonadotrophin secretion (Figure 1-11, (Cooke and Saunders, 2002; Haisenleder et al., 1994)). Inhibin consists of two subunits α and β (β_A or β_B), which results in two forms of inhibin, inhibinA ($\alpha\beta_A$) and inhibinB ($\alpha\beta_B$) (de Kretser et al., 2002; Ling et al., 1985). Both α and β subunits are synthesised in the adult testis, with greatest expression in SC and limited quantities in LC (Majdic et al., 1997). Feedback of inhibin released from the testis acts upon the anterior pituitary cells to reduce secretion of FSH independently of GnRH signalling from the hypothalamus, providing feedback between SC function and numbers, and the FSH which influences them (de Kretser et al., 2002; Ling et al., 1985; Tilbrook et al., 2001).

Steroids may exert feedback effects on gonadotrophin (LH and FSH) secretion at the hypothalamus and anterior pituitary (Shupnik and Schreihofer, 1997). Feedback of testosterone and oestrogen on gonadotrophin secretion is demonstrated in castrated male animals which display elevated LH subunit expression in pituitary, which is reduced by oestrogen or testosterone treatment (Keri et al., 1994). Action at the hypothalamus alters amplitude and frequency of the GnRH pulses that act upon the anterior pituitary gonadotropes that secrete LH and FSH (Shupnik and Schreihofer, 1997). In rats increased expression of LH mRNA caused by orchidectomy can be suppressed by both DHT and Antide (GnRH antagonist) which implicates GnRH signalling in the feedback mechanism (Fallest et al., 1995). In addition, castration in animals including rams and mice causes increased GnRH neurone activity and increased frequency of GnRH pulses. Increased GnRH neurone activity and pulse frequency is reduced by separate treatments

with testosterone or oestradiol, and is associated with altered pattern of LH secretion by gonadotropes (Jackson et al., 1991; Pielecka and Moenter, 2006). At the anterior pituitary steroids act to alter numbers of GnRH receptors expressed so altering sensitivity to these hormones (Shupnik and Schreihofer, 1997). During in vitro experiments in a gonadotrope cell line (αT3) testosterone acts to reduce expression of the gonadotrophin α-subunit thereby impacting expression of both LH and FSH, testosterone acts directly on the gonadotrope by repressing transcription of this gene (Heckert et al., 1997). Castration in rats increases expression of GnRH receptor mRNA in the anterior pituitary, in association with increased LH and FSH mRNA and serum levels, these increases can be reversed by treatment with testosterone (Kaiser et al., 1993). Pituitary cells treated with oestradiol in vitro also exhibit a reduced number of GnRH receptors (McArdle et al., 1992).

In monkeys and sheep, in which the majority of research on steroid effects at the hypothalamus and pituitary has been conducted, impact of testicular steroids is predominantly on the hypothalamus, with minimal impact on the anterior pituitary (Tilbrook et al., 2001).

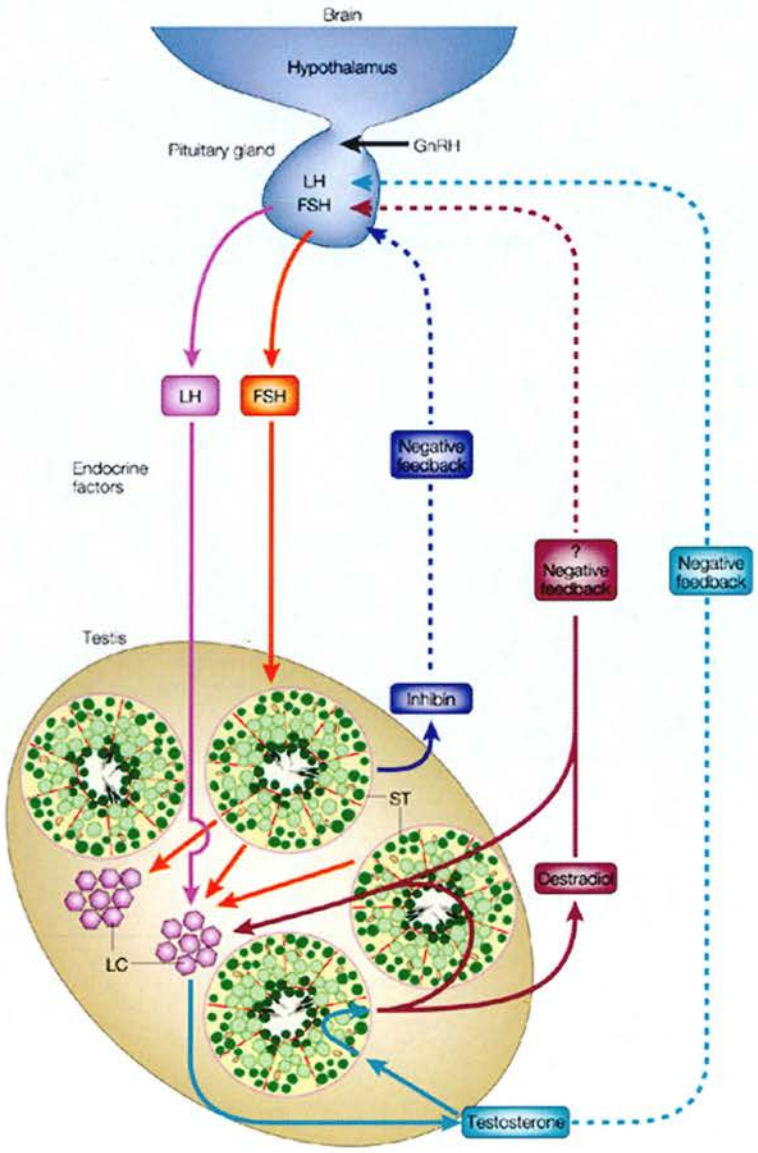


Figure 1-11: Hormone regulation within the male reproductive system. Gonadotrophins are secreted under the influence of GnRH from the hypothalamus. Gonadotrophins (LH and FSH) secreted by the pituitary act upon Leydig and Sertoli cells of the testis supporting fertility. Within the testes factors such as testosterone, oestradiol and inhibin are synthesised and signal locally as well as feeding back on the anterior pituitary inhibiting LH and FSH secretion completing the negative feedback system (taken from Cooke and Saunders, 2002).

1.3.3.2 Androgens

Expression of AR is restricted to the somatic cells within the testis and the protein has been immunolocalised to LC, PTM and SC (Bremner et al., 1994; Saunders et al., 1996; Suarez-Quian et al., 1999; Zhou et al., 2002). In rats (Bremner et al., 1994), mice (Zhou et al., 2002) and human (Suarez-Quian et al., 1996) the intensity of AR immunostaining in SC varied between stages. For example, in rats highest levels of AR protein expression were detected in SC nuclei at stages VII and VIII whereas in situ hybridisation revealed that FSH receptor mRNA was highest during stages IX and X followed by a decrease of signal intensity in stages XI-XII (Kliesch et al., 1992). There have been sporadic reports that AR are also expressed in GC (Vornberger et al., 1994) however full spermatogenesis has been achieved following transplantation of GC from mice lacking a functional AR into recipients suggesting that (at least in the mouse) expression of AR in GC is not necessary (Johnston et al., 2001).

Androgens are required for normal development of the male reproductive tract. In mice lacking a functional AR (*Tfm* or ARKO sections 1.3.3.2.1, 1.3.3.2.2 respectively) testes fail to descent and their size is dramatically reduced. In addition the prostate, epididymis, vas deferens and seminal vesicles fail to develop. Cell-selective KO of AR in different cell types within the testis has also been carried out and results point to a key role for AR expression in SC in maintenance of fertility (section 1.3.3.2.3).

The necessity of high intra-testicular testosterone (ITT) concentrations for maintenance of full spermatogenesis in rats has been investigated by depletion of LC with ethane dimethane sulphonate (EDS) (Bartlett et al., 1986) or administration of testosterone and oestradiol (T+E) implants to suppress LH secretion (O'Donnell et al., 1994), both of which deplete ITT. In EDS-treated rats, germ cell loss is first observed in seminiferous tubules at stages VII and VIII, which coincides with the tubules in which the most intense AR immunoexpression is detected in SC (Bartlett et al., 1986; Bremner et al., 1994; Hill et al., 2004). Within 4-8 days of EDS treatment, a significant increase in the incidence of pyknotic GC indicative of apoptosis is observed at stages VII and IX-XI,

testosterone supplementation can rescue the GC within 2 days, but only in stage VII tubules (Kerr et al., 1993a). EDS treatment also has an impact on protein secretion by SC (McKinnell and Sharpe, 1995), and reducing the quantity of seminiferous tubule fluid they secrete (Sharpe et al., 1994). Passage of round spermatids between stages VII and VIII of the spermatogenic cycle and the numbers of elongated spermatids present, were both dramatically reduced in the seminiferous tubules of rats treated with T+E-implants for 11 weeks to suppress ITT (O'Donnell et al., 1994). In this model, the failure of spermatogenesis in round spermatids following ITT depletion was reported to be due to stage-specific breakdown of the ectoplasmic specialisations between stages VII and VIII causing detachment of the spermatids (O'Donnell et al., 1996).

1.3.3.2.1 *Tfm* mouse

Male testicular feminised, *Tfm*, mice possess a XY genotype but a female phenotype with a vagina, a female ano-genital distance, cryptorchid testes of diminished size and no other sex-specific structures, neither male or female (Lyon and Hawkes, 1970). In *Tfm* testis spermatogenesis fails before meiosis is complete resulting in a diminished GC complement with no GC developing beyond spermatocytes, and whilst SC and LC are present, the LC appeared enlarged (Lyon and Hawkes, 1970). The genetic explanation for the *Tfm* phenotype is a single base-pair deletion in the AR gene resulting in a frameshift mutation that produces a truncated AR protein that lacks the steroid- and DNA-binding domain and consequentially the capacity to activate androgen response elements in any cell (He et al., 1991). The initial disruption in *Tfm* is the loss of AR signalling during fetal development, however the consequences of the disruption also impact upon synthesis of testosterone and DHT in the LC of adult testes (Goldstein and Wilson, 1972). The disruption of steroidogenesis in adult testes occurs at points in androgen synthesis catalysed by 17α-hydroxylase (P450c17) and 17-ketosteroid reductase (17βHSD) (seen in Figure 1-8), these deficiencies are a consequence of the requirement for androgen signalling for development of an adult population of functional LC (Murphy et al., 1994).

1.3.3.2.2 Androgen receptor knockout (ARKO)

In the ARKO mouse model AR is ablated from all tissue by virtue of crossing mice expressing Cre-recombinase under the control of the ubiquitous PGK promoter (*PGK-Cre*^{m}) with those expressing an AR gene floxed at the 2nd exon (Chang et al., 2004; De Gendt et al., 2004).

The phenotype of ARKO males closely resembles that of the *Tfm* mice with a female phenotype and no formation of epididymis, vas deferens, seminal vesicles or prostate. The testes are not located in the scrotum but remain in either the abdomen or inguinal region and are drastically reduced in size (De Gendt et al., 2004). Within the testes the lumen is absent, there are reduced numbers of SC and multiple layers of PTM cells around the perimeter of seminiferous tubules (De Gendt et al., 2004). Spermatogenesis was severely disrupted with no evidence of meiotic germ cell maturation and reduced capacity to support spermatogonial maturation in adulthood (Tan et al., 2005).

1.3.3.2.3 Sertoli cell specific androgen receptor knockout

Mice in which AR was selectively deleted only in SC, have been reported by three independent groups and reported as SCARKO (Sertoli cell androgen receptor knockout) (De Gendt et al., 2004), S-AR(-/y) (Chang et al., 2004) mice or *Ar*$^{flox(ex1\text{-}neo)/Y}$*; Amh-cre* (Holdcraft and Braun, 2004). SCARKO and S-AR(-/y) mice were both generated by crossing females with an *AR* allele floxed at the second exon of the gene with male mice that express Cre-recombinase under the control of the *AMH* promoter to ensure SC-specific deletion (De Gendt et al., 2004). In the third model a hypomorphic allele was generated by floxing exon 1 of *AR*, which resulted in reduced expression of AR in SC and other cell types in the absence of Cre recombinase. Androgen receptor expression was ablated specifically in SC by crossing mice bearing the floxed *AR* gene with mice expressing *AMH-Cre recombinase.* In these mice germ cells completed meiosis, in contrast to other SC-specific AR knockout models, but development of round spermatids into elongate spermatids failed. In addition serum testosterone was raised in the *Ar*$^{flox(ex1\text{-}neo)/Y}$*; Amh-cre* mice (Holdcraft and Braun, 2004).

In contrast to ARKO mice, in SCARKO mice testes descended into the scrotum and the epididymis, vas deferens, seminal vesicles and prostate developed normally although testis size was reduced compared to wild-type littermates (Chang et al., 2004; De Gendt et al., 2004). Spermatogenesis in SCARKO mice reached a more advanced stages than in ARKO animals, but still was not completed, due to failures in meiosis between stage VI and XII of the cycle. Although a few GC developed as far as becoming round spermatids no elongate spermatids were detected, these changes were associated with increased germ cell apoptosis and grossly reduced expression of genes specific for late spermatocyte or spermatid development (De Gendt et al., 2004). The size of the SC population in SCARKO mice is not reduced compared to wild-type testes, despite the lack of direct androgen signalling on SC (De Gendt et al., 2004; Tan et al., 2005). Expression of markers of SC maturation was normal in the SCARKO (Tan et al., 2005) and although FSH levels were 34% higher, circulating levels of LH and testosterone were normal (De Gendt et al., 2004). The pattern of GC loss observed in S-AR(-/y) mice appeared identical to that in SCARKO (Chang et al., 2004), however in contrast to SCARKO mice, elevated levels of LH and testosterone and delayed SC maturation were reported.

An array was conducted on SCARKO testes to identify potential androgen-responsive genes, and the SC protein Rhox5 was identified amongst several differentially regulated genes (Denolet et al., 2006a) (discussed in section 1.4.3). Within the testes of SCARKO mice LC were also affected, although the number of LC were normal at 12 days post partum, at later ages the LC population was depleted by >40%. In addition, these adult LC were enlarged and contained more lipid droplets and mitocondria than in wild-type testes (De Gendt et al., 2005). These studies on SCARKO testes reveal that androgen signalling in SC has consequences not just for GC development through spermatogenesis, but also development of the LC population, potentially via PDGF-A and/or oestrogen signalling (De Gendt et al., 2005).

1.3.3.3 Oestrogens

Aromatase, the enzyme required for oestrogen synthesis, is encoded by the *Cyp19* gene (Akingbemi, 2005), and is expressed in rodent testes in LC, immature SC and some but not all adult GC (Levallet et al., 1998; Rosselli and Skinner, 1992; Turner et al., 2002). In adult rats and mice ERα has been localised to LC (Pelletier et al., 2000; Zhou et al., 2002), the epithelial cells lining both the epididymis and efferent ductules that link the epididymis to the rete testis, but not to SC or GC (Fisher et al., 1997; Hess et al., 1997b; Sar and Welsch, 2000; Zhou et al., 2002). Positive immunoexpression for ERβ has been detected in somatic and germ cells in the testes of mice, rats, primates and humans (Saunders et al., 1997; Saunders et al., 2001; van Pelt et al., 1999; Zhou et al., 2002), including fetal GC of mice and rats (Jefferson et al., 2000; van Pelt et al., 1999). In rats, protein and mRNA expression was detected in all type A spermatogonia, though expression was weak, and was not detected in intermediate A-type or B-type spermatogonia. ERβ was not expressed again until pachytene spermatocytes during stages VII-XIV and round spermatids in stages I-VIII (Shughrue et al., 1998; van Pelt et al., 1999). In mice, ERβ protein was detected in all stages of spermatogonia and was abundant in spermatocytes not undergoing meiotic division (Jefferson et al., 2000; Zhou et al., 2002).

1.3.3.3.1 Aromatase KO

Aromatase knockout mice (ArKO) were generated by introducing the *Neo* gene into exon IX of the *Cyp19* gene (Fisher et al., 1998; Robertson et al., 1999). Male ArKO mice were initially fertile with a normal testis structure. However within a year of birth spermatogenesis was disrupted and although numbers of spermatogonia and spermatocytes were not reduced numbers of round and elongate spermatids were diminished (Robertson et al., 1999). The loss of spermatids was not associated with reduced SC numbers or change in fluid balance within the tubules, which did not differ significantly from wild types mice of the same age. GC loss was associated with increased apoptosis, abnormal acrosome development and sloughing of spermatids from

the seminiferous epithelium (Robertson et al., 1999). The epididymes of ArKO mice contained few or no sperm at a year old, and they were infertile in mating studies (Robertson et al., 1999). In younger animals, fourteen weeks old, reduced fertility (50% success) was observed, the spermatozoa produced were less motile and mating behaviour was disrupted (Robertson et al., 2001) GC loss was partially rescued by feeding animals with a diet rich in phytoestrogens (Robertson et al., 2002).

1.3.3.3.2 ERα knockout

Mouse lines with disruption to ERα have been generated, the first involves disruption of the gene's reading frame by insertion of the *Neo* gene into exon 2 of *ERα* (Lubahn et al., 1993). A second ERαKO was generated by excising exon3 of *ERα* using loxP sites either side of the exon and cre-recombinase expression, resulting in a truncated protein (Dupont et al., 2000). In adult male ERαKO mice there is a late onset phenotype with a reduction in testicular weight associated with a dramatic reduction in epididymal sperm count (Eddy et al., 1996; Lubahn et al., 1993). Disruption of *ERα*, results in dilated lumens in the seminiferous tubules and rete testis within twenty days of birth, initially this does not affect spermatogenesis, however ten weeks after birth spermatogenesis and sperm count began to decline (Dupont et al., 2000; Eddy et al., 1996). The dilation of the tubule lumens appears to be as a secondary consequence of reduced fluid reabsorption in the efferent ductules (Hess et al., 1997a). Not only were the numbers of sperm reduced, their motility and capacity for *in vitro* fertilisation was also impaired (Eddy et al., 1996).

1.3.3.3.3 ERβ knockout

The first ERβKO mice were generated by two different laboratories who introduced the *Neo* gene, in the reverse or correct orientation respectively, into exon 3 of the *ERβ* gene disrupting its function (Dupont et al., 2000; Krege et al., 1998). Investigations of fertility and testis morphology in both ERβKO mouse lines revealed that these animals are fertile and exhibit no disturbance to form or function of the testes (Dupont et al., 2000; Krege et al., 1998). There were reports of hyperplasia in the collecting ducts of the prostate and bladder in one study (Krege et al., 1998), but they were not found in the mice of the second study (Dupont et al., 2000). A later study using ERβKO mice, reported an increase in the population of gonocytes present during the neonatal period by as much as 50%. The increase in gonocyte numbers during the early neonatal period was reported to be due to decreased apoptosis of the cells, accompanied by a less significant increase in proliferation. The altered behaviour of the gonocytes may reflect a delay in their maturation. The effect of oestrogen via ERβ is proposed to be directly on the GCs (Delbes et al., 2004). In a study using the more robust stratergy of floxing the *ERβ* allele, mice lacking functional ERß were infertile although there was no disturbance to the testicular morphology and accessory tissue such as the prostate were also not adversely affected (Antal et al., 2008).

1.3.3.3.4 ERαβ double knockout

Heterozygous (*ER* $\alpha^{+/-}ER\beta^{+/-}$) mice were generated by crossing two mouse lines, the first bearing a disrupted *ERα* and the other a disrupted *ERβ* allele. Inbreeding between the $ER\alpha^{+/-}ER\beta^{+/-}$ mice generated the ERαβKO mice (Couse et al., 1999; Dupont et al., 2000). The consequences of knocking out both ERα and ERβ were similar to those in ERαKO mice, the testes remained intact but there was swelling within the seminiferous tubules and rete testes. Spermatogenesis was present but incomplete and the concentration and motility of sperm in the epididymis were reduced, a phenotype that mirrored that in ERαKO (Couse et al., 1999; Dupont et al., 2000).

1.4 Androgen responsive genes identified in SC

Despite the considerable evidence of stage dependent expression of AR in SC, and detrimental effects on GC development of disrupting production or action of androgens, very few androgen-responsive genes have been identified in testicular cells. The situation may change with development of new models and further analysis of existing models, such as through array analysis (described below in section 1.4.3) although analysis will often be complicated by changes in GC complement and therefore secondary effects on SC-GC interaction. There follows a description of the limited number of androgen-responsive genes identified in SC.

1.4.1 AR

The expression of AR has been shown to be regulated *in vitro* and *in vivo* by factors including androgens themselves. The expression of AR in cultured immature SC is increased two- to three-fold by treatment with androgens and FSH (Verhoeven and Caillaeu, 1988). In rats treated with EDS for 6 days, which depletes LC derived factors including testosterone, expression of AR protein is absent from all cells of the testis, including SC of stage VII tubules in which AR expression is strongest in untreated animals. The androgen dependence of AR expression in testes is demonstrated by the expression of AR in a pattern indistinguishable from untreated animals, in EDS-treated rats in which exogenous testosterone is administered to maintain intra-testicular testosterone levels (Bremner et al., 1994; Turner et al., 2001). Loss of AR expression within SC, LC and PTM cells following EDS treatment corresponds with the severe drop in T caused by LC depletion, further supporting the dependence of AR expression on androgens (Atanassova et al., 2006). Following partial restoration of T by a new population of LC, 2 weeks after EDS-treatment, AR expression was reinstated in the testis. However stage-specific expression of AR in SC only occurred when T was back within the normal adult range (Atanassova et al., 2006). High affinity binding of androgens to the AR slows the rate of receptor degradation *in vitro*, providing evidence of one mechanism by which androgens support AR protein expression (Kemppainen et al., 1992).

1.4.2 Rhox5

Rhox5, also known as *Pem*, is a reproductive homeobox gene expressed in fetal and postnatal periods that encodes a transcription factor first identified in 1990 (Lindsey and Wilkinson, 1996a; Lindsey and Wilkinson, 1996b; Sasaki et al., 1991; Wilkinson et al., 1990). *Rhox5* is expressed in male and female reproductive tissues (testes, epididymis, ovary and placenta) in rodents (Lindsey and Wilkinson, 1996b; Maiti et al., 1996). *Rhox5* is a member of cluster of 12 reproductive homeobox genes (*Rhox1 – Rhox12*) identified on the X-chromosome in mice, whose expressions are predominantly restricted to reproductive tissues. Within the testis most *Rhox* genes are expressed by SC during specific stages of spermatogenesis (Maclean et al., 2005). In addition to the *Rhox* genes identified in mice, 10 *Rhox* genes have been identified on the X-chromosome of rats and 2 *Rhox* genes (*hPEPP1* and *hPEPP2*) have been identified on human X-chromosome (Maclean et al., 2006; Wayne et al., 2002a)

Within the testis of rats and mice *Rhox5* is expressed specifically in SC, and expression begins prior to puberty (Lindsey and Wilkinson, 1996b; Maiti et al., 2001). In adult mouse testes the protein is most abundant at stages VII and VIII co-inciding with the most intense expression of AR (Lindsey and Wilkinson, 1996b). Loss of expression from the epididymis and SC of rodents in which androgen signalling is disrupted, by hypophysectomy or hypogonadism (eg Hpg mice), is consistent with regulation of *Rhox5* expression by androgens (Lindsey and Wilkinson, 1996a; Lindsey and Wilkinson, 1996b). Expression of *Rhox5* in hypophysectomised or *Hpg* mice, could be restored by treatment with LH or testosterone (Lindsey and Wilkinson, 1996b).

The *Rhox5* promoter contains two elements; the proximal promoter regulates expression in testes and epididymis whilst the distal promoter is associated with expression in other tissues and plays a less significant role in testis expression (Maiti et al., 1996; Sutton et al., 1998). The proximal promoter is androgen responsive, and contains two response elements (described in section 1.3.2.1) that are highly specific to androgen receptor binding, and not stimulation by other steroids (Barbulescu et al., 2001). In mice

expression in the testis is regulated from the proximal promoter from nine days post-partum that is just after expression of AR can be detected in SC (Tan et al., 2005) and the distal promoter has a limited function. In rats the distal promoter regulates *Rhox5* expression from twelve to thirty days after birth but thereafter expression appears dependent on the proximal promoter (Sutton et al., 1998).

The androgen sensitivity and stage-specific expression of Rhox5 suggest a role in translating androgen signalling at SC into a response in GC development. Two mouse models have been developed, in the first Rhox5 is expressed at all stages of the spermatogenic cycle, the second model is a *Rhox5* null mutant (Maclean et al., 2005; Wayne et al., 2002b). When Rhox5 is expressed in SC at all stages there is an increase in DNA strand breakage at two specific steps in spermatogenesis. In pre-leptotene spermatocytes double-strand breaks occur and in step 9 and 10 round spermatids single-strand breaks occur (Wayne et al., 2002b). Both these steps are associated with remodelling of DNA and chromatin for entry into meiosis and condensation of the nucleus for spermatid elongation, and occur during stages VII and VIII when Rhox5 is normally expressed. The DNA damage does not affect fertility or spermatogenesis because the damage is short-lived and repaired by stage XI in both cases. These effects could reflect a role for Rhox5 in dynamic changes to DNA and chromatin (Wayne et al., 2002b). *Rhox5* null mice have reduced fertility due to reduced numbers of round spermatids and elongate spermatids at stage VII and elongated spermatids in the final stages of differentiation (Maclean et al., 2005). The frequency of GC apoptosis is increased, and occurs prematurely in spermatocytes in stages VIII-XI. Fertility was further reduced by increased proportions of sperm in the epididymis whose motility is deficient (Maclean et al., 2005).

1.4.3 Further androgen responsive genes

An array on gene expression within the d10 testes of wild-type (WT) and SCARKO mice revealed forty genes which were differentially expressed, by at least two-fold,

between these mouse lines. The androgen-responsiveness of nine of these genes were further analysed by RT-PCR using testes of prepubertal mice treated with androgen (testosterone proprionate, TP) or anti-androgen (flutamide), and in untreated (control) testes. The genes *Rhox5*, *Eppin*, *Drd4*, and *Gpd1* were significantly reduced after three days anti-androgen treatment, the expression of these and the further five genes (*Galgt1*, *Tsx*, *Tubb3*, *PCI*, and *Tpd52l1*) were significantly reduced after five days treatment. All nine genes were predominantly expressed in the tubulular compartment in the testes. The expression pattern of *Eppin*, *Drd4*, and *Gpd1* during prepubertal development in both WT and SCARKO mice strongly paralleled that of the established androgen-reponsive gene *Rhox5* (see section 1.4.2), when assessed by gene array and RT-PCR. Differential expression of the serine protease inhibitors (*tPA* in addition to *Eppin* and *PCI*), cell adhesion molecules (*Cldn11*, *Emb*, and *Dsc2*), cytoskeletal components (*Actin3* and *Scin*, in addition to *Tubb3*), and members of the extracellular matrix (*Col4a6*, *Thbs1*, and *Bcan*), were also identified as differentially expressed by gene array between SCARKO and WT mice during the prepubertal period. The expression of all but *Scin*, *Bcan* and *Dsc2*, were reduced in the SCARKO testes (Denolet et al., 2006a). Comparison of gene expression in adult WT and SCARKO mouse SC in another study, also reports significant reduction in expression of *Rhox5*, plus *Aquaporin8*, *Tight junction protein 1*, *Gata1*, *Espin*, *solute carrier 7a4* and *solute carrier 38a5*, all of which are therefore putative targets of androgen-regulation (Abel et al., 2008).

1.5 Studies using isolated Sertoli cells *in vitro*

1.5.1 Primary and immortalised Sertoli cell cultures

Studies of SC function have also been performed using both primary cell cultures and immortalised cell lines. Generation of primary cultures of isolated SC is a complex procedure which is typically carried out using mice or rats between days 18 and 21 (i.e. before full spermatogenesis is established) and a protocol is described by Karl and Griswold (Karl and Griswold, 1990). Although these cultures contain a purified population of SC there are also contaminating cells, specifically peritubular myoid cells,

which under certain culture conditions can overgrow the SC within a few days (Karl and Griswold, 1990). AR expression is not maintained in the primary SC for more than a few days *in vitro* (Denolet et al., 2006b). On day 3 AR protein is reported to exhibit cytoplasmic and nuclear localisations expected in the absence or presence of testosterone respectively (Nakhla et al., 1984). Primary cultures of SC are only of limited use because they do not maintain their specialised phenotype beyond six days and primary adult SC do not divide (Skinner and Griswold, 1982; Steinberger and Jakubomaik, 1992).

Due to the limitations of primary cell cultures a number of immortalised SC-lines have been prepared. These include the Tm4 (Mather, 1980), MSC-1 (Peschon et al., 1992), and SK11 (Walther et al., 1996) cell lines. Both Tm4 cells and SK11 cells are derived from immature mice at postnatal day 11-13 and day 10 respectively (Mather, 1980; Walther et al., 1996). Immortalised cell lines, including SC, can be derived from transgenic animals expressing SV40 large T-antigen. Both the SK11 and MSC-1 lines were derived from such animals (Jat et al., 1991; Peschon et al., 1992), but Tm4 cells immortalised spontaneously in culture (Mather, 1980).

1.5.2 SK11 cell line

The SK11 immortalised cell line consists of SC derived from the *H-2K^b-tsA58* transgenic mouse, which expresses the temperature-sensitive large T-antigen (*tsA58*) under control of the *H-2K^b* promoter in cells throughout its body (Jat et al., 1991). When cells derived from this mouse are cultured at 33°C, termed the permissive temperature, the large T-antigen remains active and immortalises the cells. When the cells are cultured at the non-permissive temperature, 39.5°C, the large T-antigen is inactivated and proliferation ceases (Jat et al., 1991).

The SK11 cell line was one of several SC lines derived from the testes of prepubertal *H-2K^b-tsA58* mice ten days post-partum (Walther et al., 1996). In SK11 cells cultured at 39.5°C senescence and altered cell morphology occurred within two days, in addition

gene expression becomes similar to that of differentiated SC *in vivo* (Walther et al., 1996; Walther et al., 1997). SK11 cells at both temperatures expressed *α-inhibin*, *β-actin*, *SGP-1*, *SGP-2*, *AMH*, *Steel factor* (*kit ligand*), *transferrin*, *SF-1*, *GATA-1*, *aromatase*, *AR*, *ERβ* and *FSH receptor* mRNAs (Sneddon et al., 2005; Walther et al., 1996; Walther et al., 1997). The changes in gene expression between permissive and non-permissive temperatures include increased *SGP-2* and *transferrin* expression, and decreased expression of *Steel factor* (*kit ligand*) (Sneddon et al., 2005; Walther et al., 1996; Walther et al., 1997). SK11 cells are also reported to be responsive to testosterone and forskolin, with expression of *transferrin*, *Dax-1*, and *aromatase* increased by treatment with one or both (Walther et al., 1997). A previous study demonstrated the presence of functional AR and ERβ that were able to stimulate androgen and oestrogen response elements respectively in the prescence of DHT and E2 and to up-regulate expression of their own mRNAs. AR and ERß proteins were localised to the nuclei of SK11 cells cultured at 33°C and 39°C, and expression of AR, but not ERβ, appeared greater at 39°C (Sneddon et al., 2005).

1.6 Adenovirus

Human Adenoviruses are non-enveloped icosahedral DNA viruses that are separated into six species (A-F), some serotypes are commonly used as vectors for introduction of genetic material into cells *in vivo* and *in vitro* (Goncalves and de Vries, 2006). The use of adenoviruses as vectors requires genetic modification to optimise their use (Bett et al., 1994).

1.6.1 Wild-type virus

The wild-type adenovirus consists of the viral genome encapsulated with the capsid head, attached to the surface of the capsid fibre proteins. The structure of an adenoviral particle is shown in Figure 1-12. Together pII (hexon) and pIII (penton) proteins constitute the key constituents of the viral capsid shell. The fibres attached to the capsid consist of pIV protein trimers, whose C-terminal globular 'knob' domain interacts with

the adenoviral, CAR, receptor (Henry et al., 1994; Maizel et al., 1968). Inside the capsid the viral genome is bound to polypeptide pVII (Brown et al., 1975) and terminal protein (Rekosh et al., 1977), this nucleoprotein complex is bound to the inside of the capsid by polypeptide pV (Goncalves and de Vries, 2006; Matthews and Russell, 1998).

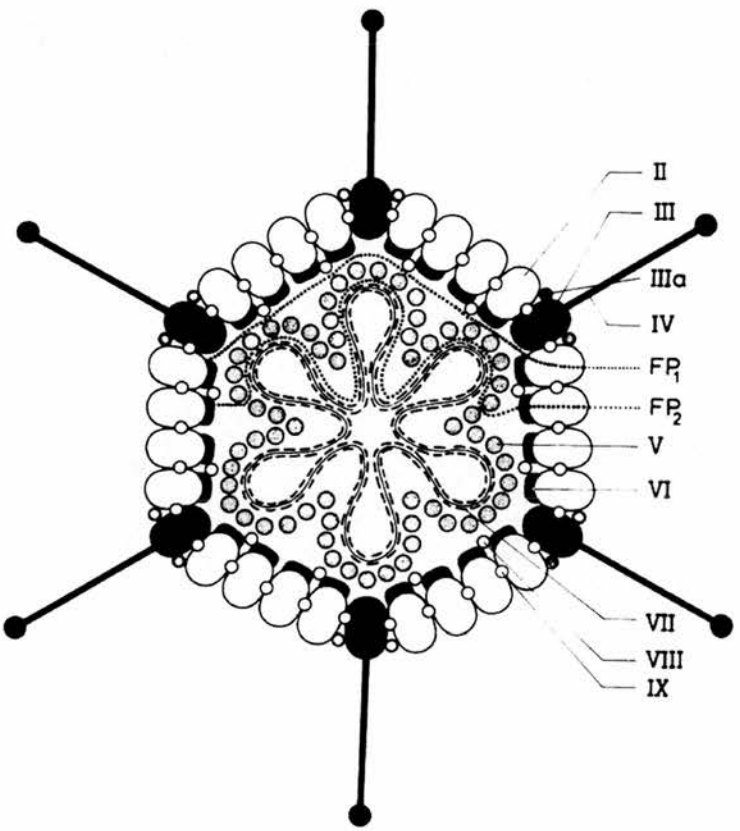


Figure 1-12: Structure and protein constituents of adenoviral particle. Roman numerals indicate location of each polypeptide within the particle structure. Key components are pII and pIII of the capsid, pIV fibres bound to the capsid, and pV and pVII enclosed in the capsid associated with the viral genome (taken from Brown et al., 1975).

During infection of host cells the viral genome is expressed in two phases, that occur before (early phase) and after (late phase) viral genome replication. In the early phase transcription units *E1-4* are expressed, expression of unit *E1A* is absolutely required for subsequent expression of other early gene units (Berk et al., 1979; Jones and Shenk,

1979; Nevins, 1981). The *E1B* and *E4* repress synthesis of host cell proteins (Babiss and Ginsberg, 1984; Halbert et al., 1985); *E2* induces DNA synthesis by contributing DNA-binding protein, terminal protein and DNA polymerase to a pre-initiation complex (de Jong and van der Vliet, 1999). Unit *E3* disturbs the host cell immune response, for example by interfering with viral antigen presentation by MHC I at the host cell surface (Andersson et al., 1985; Goncalves and de Vries, 2006). During the late phase, genes under control of the *E2* late promoter and major late promoter (MLP) are expressed. MLP genes (*L1-5*) primarily encode the proteins of the capsid, fibres and nucleoprotein complex. The products of the early and late phase combine in the host cell nucleus yielding new viral particles (Goncalves and de Vries, 2006).

1.6.2 Modifications for laboratory use

Species C adenoviruses of serotype 2 or 5 are the most commonly used as vectors for introduction of transgenes into cells *in vivo* and *in vitro*, they are modified from wild-type adenoviruses to enable them to carry significant quantities of foreign DNA and to prevent lysis of the infected cells (Goncalves and de Vries, 2006; Volpers and Kochanek, 2004)). Removal of the *E1* domain increases the vector's capacity for transgenes, and also prevents expression of the other early viral units (*E1-4*), which in turn prevents productive viral cycles and cell lysis (Goncalves and de Vries, 2006; Jones and Shenk, 1979)). The removal of *E1* from the viral genome means that the vectors have to be propagated in a cell expressing the *E1* genes, to provide the essential *E1*-encoded products required for a productive viral cycle. HEK293 cells are a frequently used helper-cell line that expresses 12% of the left and 10% of the right hand components of the human adenovirus group 5 genome, and support the proliferation of *E1*-deficient adenoviruses (Goncalves and de Vries, 2006; Graham et al., 1977).

Substitution of the *E1* domain for the transgene provides the vector with a capacity of nearly five kilobases, and an additional substitution within the *E3* domain can raise the capacity to at least 7.5 kilobases (Bett et al., 1994; Haj-Ahmad and Graham, 1986). The

absence of *E3* does not impact on the function of the virus *in vitro* (Bett et al., 1994) (Haj-Ahmad and Graham, 1986), however a role is reported for *E3* in limiting immune response to infection of cells *in vivo* (Andersson et al., 1985; Goncalves and de Vries, 2006)).

Second-generation adenoviral vectors have been developed in which *E1* and *E2* or *E4* domains have been ablated or deleted. These vectors require different culture conditions or helper-cell lines, expressing more viral products than HEK293 cells, to compensate for the loss of additional early genes during amplification of the vector (Engelhardt et al., 1994; Gao et al., 1996; Wang et al., 1997). The second-generation vectors were developed to improve the duration of transgene expression in target tissues and to reduce immune responses. *E1*-deleted vectors are associated with cytotoxicity, virus-specific immune responses and inflammation, that are less severe when *E1-E2/4* deleted vectors are used (Engelhardt et al., 1994; Gao et al., 1996; Wang et al., 1997). As a consequence, the transgenes carried by these vectors are expressed up to three months after infection, compared to around three weeks with *E1*-deletion alone (Engelhardt et al., 1994; Wang et al., 1997).

A final form of adenoviral vectors are called helper-dependent or 'gutted' because they contain none of the adenoviral genome and require co-transfection with a helper-virus when amplifying the gene *in vitro*. The helper virus expresses all the viral genes required to package the genetically modified viral genome of the vector, but is modified so the competent genome is not incorporated into the viral particles (Parks et al., 1996). The gutted vectors have a capacity for much larger transgenes than first or second-generation adenoviral vectors, and eliminate expression of adenoviral proteins reducing the immune response to infection. The duration of transgene expression in a gutted compared to a first-generation vector is much greater with expression associated with gutted vectors remaining two months after infection (Morsy et al., 1998).

1.6.3 Coxsachie B virus and adenovirus receptor (CAR)

Type 2 and 5 adenoviruses, of species C, both bind to the same receptor which is embedded in the plasma membrane of cells (Philipson et al., 1968). The receptor (CAR) has been shown to be a common binding site for both type C adenoviruses and coxsackie B virus and is a 46 kDa glycoprotein expressed in mice and humans. Comparison of the human CAR protein's amino acid sequence with that of the mouse CAR receptor revealed between eighty and ninety percent homology; two extracellular immunoglobulin-like domains were also identified (Bergelson et al., 1997; Bergelson et al., 1998; Tomko et al., 1997).

Expression of human *CAR* mRNA was detected in pancreas, brain, heart, small intestines, testis, and prostate, plus small amounts in liver and lungs. In mouse concentrations of mRNA were highest in liver, with weaker expression in kidney, heart, lungs, and brain (Tomko et al., 1997). Within the testes of rats, CAR was identified at the BTB and ES, at sites of SC-SC and SC-GC interaction, and *CAR* mRNA and protein are detected in both SC and GC (Wang et al., 2007). In mice SC and LC were immunopositive for CAR and strong immunostaining was associated with elongate spermatids, weaker staining was observed with less mature GC (Mirza et al., 2006; Peters et al., 2001). The expression of CAR in ectoplasmic specialisations is strongest when associated with elongated spermatids prior to release into the lumen at stage VIII, weaker staining is associated with round spermatids and less well developed elongating spermatids (Wang et al., 2007). Expression of CAR can be detected in SC and spermatogonia as early as fifteen days post partum, prior to the development of BTB. Expression of CAR has been detected in spermatocytes as well (Wang et al., 2007).

1.6.4 Adenoviral Infection

1.6.4.1 Mechanism

Adenoviral particles interact with their target cell at two sites in the plasma membrane, the CAR receptor and integrins. Binding to the CAR receptor is via the trimeric pIV

protein fibres attached to the viral capsid as described above (Philipson et al., 1968). There are a number of different integrin heterodimers consisting of combinations of α and β subunits, including αVβ3, αVβ5, αVβ1, α3β1, and α5β1, which have been implicated in internalisation of adenoviral particles (Davison et al., 1997; Davison et al., 2001; Salone et al., 2003; Wickham et al., 1994; Wickham et al., 1993). The cell surface integrins frequently interact with Arg-Gly-Asp amino acid sequence (RGB domain) in the capsid's penton protein (Wickham et al., 1993), although some, such as integrin α3β1, can interact with other targets in the penton protein (Salone et al., 2003). Viral particles enter target cells very rapidly by receptor-mediated endocytosis via clatherin-coated pits and the vesicles (Chardonnet and Dales, 1970; Svensson, 1985; Varga et al., 1991). The process of endocytosis is mediated by integrins including αVβ3 and αVβ5 (Wickham et al., 1993). Once endocytosis has been induced the viral particles must escape from the vesicles into the cell cytoplasm by disrupting the endosome (FitzGerald et al., 1983). The increased endosome permeability associated with its disruption is dependent upon presence of αVβ5 in the cell membrane for interaction with the penton base (FitzGerald et al., 1983; Wang et al., 2000). The reduction in endosomal stability is due to signals from the cell surface induced by viral attachment (Meier et al., 2002)and is due to reduced pH (~5) within the vesicle (Blumenthal et al., 1986; Prchla et al., 1995; Seth et al., 1984; Wickham et al., 1994).

Following release from the endosome pathway viral particles are transported through the cell cytoplasm towards the nucleus and towards the cell periphery. Transport is along the cells microtubules and in untreated cells in predominantly towards the nucleus resulting in viral particles accumulating at the nuclear membrane. The movement towards the nucleus is termed 'minus-end-directed' and requires the dynein/dynactin motor for progress along the microtubules and directly attaches the adenoviral particle to the tubules (Kelkar et al., 2004; Suomalainen et al., 2001; Suomalainen et al., 1999). The final process in adenoviral infection is entry into the nucleus to undergo expression of the adenoviral genome as described in section 1.6.1. As the viral particles progress through the cell from the cell surface and eventually into the nucleus the virus

progressively losses constituent proteins until finally before the viral genome enters the nucleus it dissociates from protein pV (Greber et al., 1993). Import of adenovirus type 2 DNA begins with the virus binding to CAN/Nup214 in the nuclear pore complex via its fibrils. The capsids of the bound viral particles breakdown but protein fragments remain bound to the genome these DNA-protein units associated with histone H1, Impβ and Imp7 and are imported into the nucleus (Trotman et al., 2001).

1.7 Conclusions

Interference with androgen and/or oestrogen signalling by loss of steroids or functional receptors disrupts fertility in rodent models and human patients, however the mechanisms for these effects are not clear. Sertoli cell specific knockout of AR in mice, and other studies, emphatically show the SC's involvement in support of spermatogenesis, so further investigation of this cells' responses to steroids is merited. To date very few steroid-regulated genes have been identified in SC. SC are the only somatic cell within the seminiferous epithelium the bulk of which is made up by germ cells. Studies on this cell type therefore remain challenging. An *in vitro* model, such as the SK11 cell line, isolates the SC from the complex environment of the testis, allowing the study of steroid response in the absence of other confounding factors, such as interactions with GC and indirect effects due to the responses of other testicular cells to the experimental manipulations. Whilst the *in vitro* approach seeks to avoid the complexities of the testis, an isolated SC is not physiologically normal as it lacks the cyclic changes and interactions that it would undergo *in vivo*. Current methods for specific knockout of receptors in SC (e.g. SCARKO mice) result in development of SC in the absence of hormone action with potential consequences for their prepubertal development. The potential for a method of knockdown of AR (or ERβ), after pubertal development is complete, using RNAi delivered by adenoviral vector is therefore an attractive option. A technique already employed to disrupt intra-testicular testosterone by administration of EDS to rats, also allows 'normal' development of testes before androgen signalling is disrupted by LC depletion. Although the impact of EDS treatment is broader than simply depleting testosterone, the ease with which testosterone

can be reintroduced makes this a favourable *in vivo* model for study of testosterone function within testes.

1.8 Aims

Use the SK11 *in vitro* cell model, to extend the investigation of androgen and oestrogen responsiveness of SC, and to identify genes which are differentially regulated under control of these steroids. Validate androgen-responsiveness of genes identified in published studies using the SK11 cells. It was hoped that genes identified as androgen or oestrogen responsive in the SK11 cells may be associated with control of spermatogenesis by SC, revealing mechanisms for regulation of spermatogenesis by steroids.

To develop a method for introduction of RNAi constructs specifically into SC *in vivo* and *in vitro*, using adenoviral constructs, with the intension of knocking out AR and ERβ expression separately in SC. Undertake a pilot study to determine efficiency of construct expression, cell-specificity of infection, and impact of infection on cell and tissue function.

To investigate mRNA and proteins expression of genes identified as androgen-responsive in previously published studies, using the EDS-treated rat model to confirm and validate these results. Genes associated with ectoplasmic specialisations, gap junctions and cytoskeleton will be examined.

The overall aim of the these studies was to deverlop and extend the methods for examining Sertoli cell function in regulation of spermatogenesis by androgens and oestrogens, and to further validate impact of steroids on expression of genes and proteins identified previously as androgen-responsive.

2 Materials and Methods

2.1 Animals

2.1.1 Animal experimentation

Animal experimentation was performed under project licence PPL 60/3544 in adherence to the UK Home Office Animals (Scientific Procedures) Act 1986. Animals were housed in 12hour:12hour (light: dark) conditions, 22/23°C, 50% humidity with access to food and water *ad libitum*.

2.1.2 Control tissues

Tissues (testis, prostate, epididymis, and kidney) of wild-type mice from mating of *Dazl*$^{Tm1hgu/m1hgu}$ heterozygous mice were used as a source of control protein and RNA in studies of cultured Sertoli cell differentiation and response to steroid treatment (Chapters 3). Adult wild-type mice on a *C57 BL/6* background were used for *in vivo* infection of Sertoli cells by intra-testicular injection with the contralateral testis as the paired control (chapter 4).

Adult Wistar rat testes injected with dimethylsulphoxide/water (1:3; v/v) and arachis oil were collected for use in PCR, Western blots and immunohistochemistry as control tissue in studies on EDS-treated rats (Chapter 5).

2.1.3 Tissue Recovery

Animals used in these studies were killed by schedule 1 methods (inhalation of CO_2 and cervical dislocation) and tissues were immediately dissected out of the animals and onto dry ice for RNA or protein extraction (sections 2.3.1 and 2.4.1), or into fixative (section 2.5.1) for immunohistochemistry. The tissues collected from treated animals were

limited to the testes, efferent ducts and epididymis. Testes, prostate, epididymis and kidney were collected from wild type mice.

2.2 Sertoli cell cultures

An immortalised Sertoli cell line (SK11) was used throughout the studies. The SK11 cell line was derived by Walther et al. at Institute of Hamburg (Walther et al., 1997) from Sertoli cells isolated from the testes of 10 day old transgenic mice, *H-2K^b-tsA58*, expressing the temperature sensitive SV40 T antigen (Jat et al., 1991). Incubation at temperatures permissive of T antigen activity (34°C) results in proliferation of the cells, at the non-permissive temperature (39°C) the T-antigen is inactivated and proliferation stops, this is associated with changes in gene expression which parallel those of differentiated Sertoli cells *in vivo* (Sneddon et al., 2005).

2.2.1 Culture conditions

SK11 cells were incubated in 'complete' medium (section 2.8.1) in humidified conditions at either 34°C or 39°C with 5% carbon dioxide. Cells were routinely cultured in T75 flasks at 34°C to maintain sufficient numbers of cells for experimental treatments. To differentiate the cells they were incubated for 48 hours at 39°C. Prior to experimental treatments, including treatment with steroids and culture at permissive and non-permissive temperatures, cells were cultured in media free of phenol-red and containing 10% charcoal-stripped fetal bovine serum plus additional supplements for 48 hours (see section 2.8.1).

2.2.2 Passaging of cells

Cells grown in T75 flasks were passaged at 2-3 day intervals, or when cell density within culture vessels reached ~70%. 'Complete' medium was removed and cells were washed with 5 ml PBS (phosphate buffered saline) without $CaCl_2$ or $MgCl_2$ (Gibco, Paisley, UK) to remove any remaining medium. The PBS was replaced with 2 ml of 1x

0.05% (w/v) Trypsin-EDTA (Gibco) and cells were incubated for 2-4 minutes at 37°C until the cells detached from the flask's surface. Trypsin digestion was inactivated by addition of 8ml of 'Complete' media containing 10% FBS and the cell suspensions were centrifuged for 5 minutes at 115 x g to pellet the cells. For passaging of the cells, the pellet was re-suspended in a volume of 'complete' media, 1/6 of that volume was transferred to each fresh T75 flask and the total volume of media per flask was made up to 10 ml. Cells for use in studies were counted using a haemocytometer, diluted in phenol-red free 'complete' medium, and plated into culture vessels at the densities shown in Table 2-1

Table 2-1: Density of cells seeded to culture vessels.

Culture vessel	Cell density (cells/compartment)
6-well plate	$4x10^5$
12-well plate	$1x10^5$
96-well plate	$7.5x10^3$

2.2.3 Steroid treatments

SK11 cells were treated with various ligands including androgens, oestrogens and a steroid receptor antagonist. Testosterone (Sigma-Aldrich, Poole, Dorset, UK) was dissolved in 100% ethanol and serially diluted to between $1x10^{-6}$ and $1x10^{-3}$ M. DHT (Sigma-Aldrich) and the natural oestrogen 17β-oestradiol (E2, Sidma-Aldrich) were diluted in filter-sterilised DMSO to $1x10^{-2}$ M and further diluted to $1x10^{-4}$ M in sterile PBS then serially diluted from $1x10^{-8}$ M to $1x10^{-4}$ M in DMSO diluted 1/100 in PBS to maintain the concentration of DMSO present. Diethylstibestrol (DES, Sigma), a synthetic oestrogen, was serially diluted to between $1x10^{-9}$ and $1x10^{-4}$ M in DMSO diluted 1/100 in PBS as above, 5 alpha-androstane-3-beta, 17 beta-diol (3βAdiol, Sigma) was diluted in ethanol diluted 1/100 in PBS, to $1x10^{-4}$ M, and then serially diluted with the same ethanol/PBS solution to concentration between $1x10^{-8}$ M and $1x10^{-4}$ M. ICI

182,780 (Tocris Cookson), is an oestrogen receptor antagonist, and was diluted in neat DMSO, to a concentration of $1x10^{-3}$ M. All ligands and antagonist were used at a further 1/1000 dilution in culture media for treatment of the SK11 cells, to give concentrations in the range of $1x10^{-12}$ M to $1x10^{-6}$ M.

2.3 Polymerase Chain Reaction

2.3.1 RNA extraction and quantification

The lysis and homogenisation of both cells and tissue was by centrifugation through QiaShredders (Qiagen, Crawley, West Sussex, UK) in the presence of denaturing guanidine-thiocyanate buffer to prevent degradation by RNases that were released when cells were lysed. Cells were removed from culture vessels suspended in guanine-thiocyanate buffer using cell scrapers and were immediately homogenised in Qiashredders. Tissue was snap frozen and ground under liquid nitrogen in an ice-cold mortar and pestle and up to 30 mg of the powdered tissue was suspended in guanidine-thiocyanate buffer to be lysed and homogenised in the QiaShredders according to the manufacturer's instructions.

RNA was extracted from both tissue and cells using RNeasy extraction kits (Qiagen). During extraction, RNA present in lysed and homogenised samples, was bound to a silica membrane in the presence of a high-salt buffer and ethanol. Contaminants within the samples are removed by a series of three washes, and the purified RNA sample is finally eluted from the membrane in RNase-free water, achieved through centrifugation. During the procedure samples bound to the silica membrane were treated with DNase (Qiagen) to degrade any DNA within in the sample.

All tubes and pipette tips used in the processing of the cells and tissue were RNase-free and pipettes, spatulas and bench surfaces were treated with RNase Zap (Ambion, Huntingdon, UK) to eliminate RNase activity.

RNA concentrations were determined using either the Nanodrop ND-1000 spectrophotometer (LabTech International, Ringmer, East Sussex, UK) or 2100 Bioanalyzer and RNA nanochip system (Agilent Technologies, South Queensferry, West Lothian, UK). Briefly, the 2100 Bioanalyzer and nanochip were used to quantify RNA concentration and sample quality in a 1 µl sample, fluorescently labelled samples were forced electrophoretically through separate microchannels and detected to provide gel-like images and electropherograms displaying bands and peaks representing the *18S* and *28S* ribosomal RNA from which total RNA quantity and quality were assessed according to the manufacturer's instructions. The Nanodrop ND-1000 also assesses nucleic acid concentration and quality from a 1 µl sample. The sample was pipetted onto the lower pedestal of the machine and light absorbance by the sample (OD) was measured for wavelengths of 260 nm and 280 nm. The absorbance at 260 nm is proportional to the RNA concentration, the absorbance at 280 nm detects the amount of protein contamination and therefore the 260:280 absorbance ratio gives an estimate of the quality of the sample. A sample with an RNA concentration of 40 ng/µl has an OD_{260} of 1, and a pure sample of RNA has a 260:280 ratio of 2. A 260:280 ratio lower than 2 indicates contamination of the sample by proteins or phenol (Sambrook et al., 1989). The samples analysed on the ND-1000 were compared against a blank standard consisting of RNase free water, which was used to set zero for the OD measurements. All RNA samples were diluted in RNase-free water to a concentration of 100 ng/µl prior to use in RT-PCR and TaqMan Quantitative RT-PCR.

2.3.2 Semi-quantitative RT-PCR

Reverse transcriptase PCR was performed on RNA extracted from both cultured cells and animal tissues (section 2.3.1). cDNA was synthesised from 1 µg of total RNA diluted in 10 µl of water plus 1 µl of oligo dT (50 µM). The oligo (dT) and RNA were incubated together at 70°C for 5 minutes and then held on ice to allow binding of the thymine nucleotides to the poly-A tails of the mRNAs, until the following reagents were added:

RNase-free H_2O (Promega, Southampton, UK)	3.85 µl
20 U/µl RNase inhibitors	0.5 µl
(Applied Biosystems, Warrington, UK)	
5x reaction buffer (Bioline, London, UK)	4 µl
10 mM dNTPs (Promega)	0.4 µl
200 U/µl Bioscript Reverse transcriptase	0.25 µl
(Bioline)	
Total Volume	**20 µl**

The reaction mixture was incubated for 60 minutes at 42°C, then heated at 72°C for 10 minutes to inactivate the reverse transcriptase. The PCR reaction using cDNA prepared with oligo dT was performed by two methods. The first method used separate Taq polymerase (Bioline) and associated reagents and buffers as shown below:

cDNA	2 µl
Taq Polymerase (Bioline)	0.25 µl
5 µM Forward primer (MWG,	1 µl
Ebersberg, Germany)	
5 µM Reverse primers(MWG)	1 µl
50 mM $MgCl_2$ (Bioline)	0.3 µl
2 mM dNTPs	1 µl
10x Reaction Buffer (Bioline)	1 µl
RNase-free H_2O	3.45 µl
Total Volume	**10 µl**

In the second method the Taq polymerase, reaction buffer, $MgCl_2$ and dNTPs were supplied together as Biomix™ Red (2x). The reaction mixture was as follows:

cDNA	2 µl
Biomix™ Red (Bioline)	5 µl
Forward Primers	1 µl
Reverse Primers	1 µl
RNase-free water	1 µl
Total Volume	**10 µl**

In both methods, cDNA was amplified with the following temperature cycles:

95°C (First Denaturation)	5 minutes	
95°C (Repeated Denaturations)	1 minute	
A°C (Annealing)	1 minute	30 cycles
72°C (Extension)	1 minute	
72°C (Final Extension)	10 minute	

Although 30 cycles were used most frequently, between 25 and 35 cycles were used in instances where the gene of interest had high or low levels of mRNA expression. The annealing temperature (Labelled A°C) was dependent upon guanine and cytosine content of the primers in use, the annealing temperature for each primer pair is shown in Table 2-2, together with the primer sequences and the expected PCR product size.

ERβ mRNA expression was assessed by nested-PCR, the cDNA and PCR were prepared as described above. However, 2 µl of the products from the PCR were used as template for a second round of PCR using another pair of primers designed to amplify the product from the first reaction (Nested ERβ, Table 2-2).

Table 2-2: RT-PCR Primer sequences, with product size and annealing temperature.

Gene	5' Primer Sequence	3' Primer Sequence	Product Size (bp)	Annealing Temperature (°C)
AR	GGACCATGTTTT ACCCATCG	TCGTTTCTGCTG GCACATAG	171	56
ERβ	CCAATGTGCTAG TGAGCCG	AACTCACGGAAC CGTGCCG	393	55
ERβ (nested)	TGCTAGTGAGCC GTCC	CCAAAGATTTCC AGAATCC	348	50
GAPDH	CTGCACCACCAA CTGCTTAGC	ATGCCAGTGAGC TTCCGTTC	288	58
Rhox5	GTGGACAAGAG GAAGCACAA	TCTCCCCATCTC ACTCCAC	635	55
SGP-1	TAAGGCTAACGA GGACGTCTGC	GCCTGGACCAGA TTCTGCTCAT	354	58
SGP-2	CATCTGGCATCA TAGACACGCT	ACACAGTGCGGT CATCTTCACC	460	58

Products of the reaction were run on 2% agarose gels containing either 0.5 μg/ml ethidium bromide (Sigma) or Biotium GelRed™ (Cambridge Bioscience, Cambridge, UK) diluted 1:10,000, gels were made up with and run in 1x TAE buffer (Section 2.8.3) at 80 V. Hyperladder IV (Bioline) that produces bands at 100 bp intervals from 100 to 1000 bp was run in parallel to samples to identify the size of the products. Hyperladder 1 (Bioline) and O'GeneRuler (Fermentas, York, UK) were used less frequently. The PCR product was visualised on the gels using a GeneFlash transluminator (GRI Syngene, Braintree, Essex, UK), digital images of the band patterns were acquired and stored for analysis.

2.3.3 TaqMan Quantitative PCR

2.3.3.1 cDNA Synthesis

To quantify expression and in order to detect low abundance transcripts TaqMan Quantitative RT-PCR was employed. In contrast to the previous method cDNA was prepared in a random hexamer primed reaction and required only 200 ng of RNA per reaction:

RNA (100 ng/µl)	2 µl
10x RT buffer (Applied Biosystems)	1 µl
25 mM Magnesium Chloride (Applied Biosystems)	2.2 µl
10 mM dNTPs (Applied Biosystems)	2 µl
50 µM Random Hexamers (Applied Biosystems)	0.5 µl
20 U/µl RNase inhibitors (Applied Biosystems)	0.2 µl
50 U/µl Multiscript Reverse Transcriptase (Applied Biosystems)	0.25 µl
Total Volume	**10 µl**

Reaction mixture was incubated at:

25°C, 20 minutes
42°C, 60 minutes
95°C, 5 minutes

Quantitative PCR was conducted on the cDNA using 2 methods: the first used Assay-On-Demand™ primer-probe mixtures (Applied Biosystems) specifically designed and optimised for detection of the genes of interest in the mouse. The second system

involved Roche's Universal Probe library™ (Roche, Burgess Hill, UK) and an oligonucleotide pair specific to the gene of interest.

2.3.3.2 Principles of TaqMan™ Quantitative PCR

TaqMan™, whether by the Assay-On-Demand™ or Universal Probe Library™ (UPL) method, works by the same principle. The primers function in the same manner as in conventional PCR, binding within the gene's cDNA sequence providing the site for initiation of DNA replication catalysed by DNA (Taq) polymerase. However, the forward and reverse primers are designed to bind close together within the sequence producing a product of less than 100 bp, within which there is a binding site for a sequence-specific probe. These probes are labelled with a 5' fluorescent reporter dye and a 3' quencher in close proximity. In the Assay-On-Demand™ kits the fluorescent dye is Fluorescein (FAM™) and the quencher is 6-carboxy-tetramethyl-rhodamine (TAMRA™), in the UPL the fluorescent dye is also FAM™ and the quencher is a dark quencher dye. The probes used to detect endogenous loading controls (*18S* and *GAPDH*) are labelled with VIC fluorescent dye and the quencher TAMRA™. When the fluorescent dye and quencher are in close proximity, as they are when bound to opposite ends of the probe, the reporter dye's fluorescence is inhibited. In the process of amplifying the PCR products the nuclease activity of Taq DNA polymerase progressively cleaves nucleotides from the 5' end of the probe which releases the fluorescent marker and frees it from inhibition by the quencher and the reporter fluoresces. A summary of the process is shown in Figure 2-1. The amount of fluorescence emitted is a measure of the number of PCR products present during each cycle of PCR. By recording the time, and thus the number of PCR cycles, required to reach a threshold fluorescence level, the concentration of the original transcript relative to other samples in the same run can be calculated (Section 2.3.3.5).

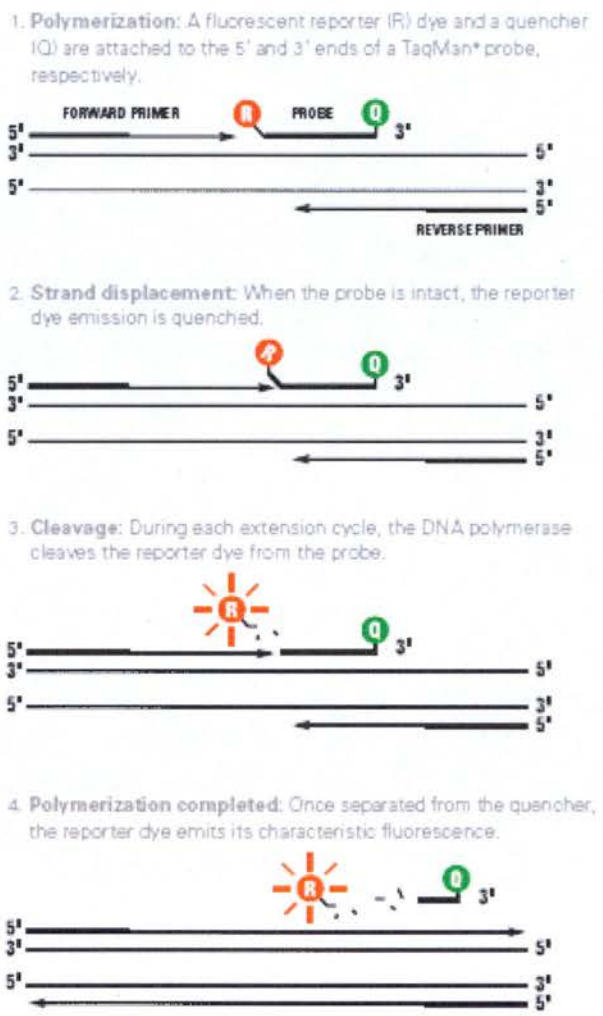


Figure 2-1: Diagram of the principle steps in Taqman real-time PCR. Diagram taken from http://www3.appliedbiosystems.com/AB_Home/applicationstechnologies/ Real-TimePCR/ TaqManvsSYBRGreenChemistries/index.htm

2.3.3.3 Assay-On-Demand™

Primer-probe combinations specific to *Rhox5* (Cat no Mm00476718_m1), *AR* (Cat no Mm00442688_m1), and *SGP-2* (Cat no Mm00442771_m1) were purchased from Applied Biosystems (Warrington, UK). Primer-probe mix to detect either the *18S* ribosomal subunit or expression of *GAPDH* (Applied Biosystems) was included as an

internal control for the amount the cDNA added in each sample. Each PCR reaction consisted of:

cDNA	7.5 µl
Primer-probe (gene of interest) (Applied Biosystems)	3.75 µl
Primer-probe (loading control) (Applied Biosystems)	1.125 µl
Master mix (Applied Biosystems)	37.5 µl
RNase-free Water	25.125 µl
Total volume	**75 µl**

As mentioned above an internal control, of primers/probes for *18S* or *GAPDH*, was included in the reaction mixture. The primers and probes for both internal loading controls were supplied and used at the same concentrations. The stock primer/probe mix consisted of:

20 µM Probe (Applied Biosystems)	106 µl
10 µM Forward Primer (Applied Biosystems)	53 µl
10 µM Reverse Primer (Applied Biosystems)	53 µl
Nuclease-free water	188 µl
Total Volume	**400 µl**

Reaction mixtures were loaded in triplicate with 25 µl per well into a 96-well MicroAmp fast optical reaction plate (Applied Biosystems) for analysis on an ABI 7500 or ABI 7900 HT Fast Real-Time PCR machine (Applied Biosystems), reactions were sealed on the plate with ABI prism optical adhesive cover (Applied Biosystems). The system was further optimised in later studies allowing the total volume per triplicate to be reduced to 45 µl, all constituents remained in the same proportions.

2.3.3.4 Roche Univeral Human Probe Library™

The Roche Univeral Probe Library™ consists of 165 probes, 8-9 nucleotides long, each with several thousand binding sites distributed across the human genome and genomes of other related organisms, including the mouse. Primers specific for a particular gene are incubated with a probe that binds to the sequence between the forward and reverse primers and is transcribed during the PCR reaction. Transcription of the sequence between the primers results in fluorescence of the 5' FAM-label (section 2.3.3.2) that is detected as a measure of gene expression.

Using the on-line Roche assay design centre, primers were designed against the genes shown in Table 2-3. The design centre provides the sequence of both the forward and reverse primers and indicates the appropriate probe to use. Primers were ordered from MWG Biotech (Ebersberg, Germany) and were diluted in nuclease-free water to a concentration of 20 µM. PCR was performed on cDNA prepared with random hexamers (section 2.3.3.1) with an internal *18S* control as in the Assay-On-Demand. The PCR reaction mixture consisted of:

cDNA	6 µl
20µM Forward primer (MWG)	0.6 µl
20µM Reverse primer (MWG)	0.6 µl
10µM Probe (Roche)	0.3 µl
18S primer/probe (Applied Biosystems)	0.9 µl
2x Faststart mastermix (Roche)	30 µl
6µM Rox dye (Roche)	4.5 µl
H_2O	17.1 µl
Total volume	**60 µl**

The reaction was run in triplicate with 20 µl per well in a 96-well MicroAmp fast optical reaction plate and run as with Assay-On-Demand™.

Table 2-3: Primer and probe sequences for use in Universal Human Probe Library Taqman

Gene	Forward Primer 5' – 3'	Reverse Primer 5' – 3'	Probe (Probe number)
AR (mouse)	CCAGTCCCAATTGTG TCAAA	TCCCTGGTACTGTCC AAACG	GGATGGAG (#58)
Cldn3 (mouse)	TGGGAGCTGGGTTG TACG	CAGGAGCAACACAG CAAGG	CTGGGCTG (#26)
Cldn11 (mouse)	TGGAGTGGCCAAGT ACAGG	GACAATGGCGCAGA GAGC	CTGGCTGG (#20)
Interleukin -6 (mouse)	GCTACCAAACTGGA TATAATCAGGA	CCAGGTAGCTATGG TACTCCAGAA	TTCCTCTG (#6)
Rhox5 (mouse)	AAATGAGCCAGTTG CTGAGG	ATCTCCTACCCCCAG GAT	CAGGAGAA (#2)
AR (rat)	TTCTTCTTCTCTGCC TCTTTTACC	AAGAAATACAACAA AACTCAAAACACA	GGCTCCTG (#92)
β3-tubulin (rat)	CAGAGCCATTCTGG TGGAC	GCCAGCACCACTCT GACC	GAGCCTGG (#116)
Espin (rat)	TGCAGTGGCTCACA C	GACCGTGGCACCAG AGTTAT	GGTGGCTG (#83)
Rhox5 (rat)	AGCGCATTTTGCTAA GCAGT	TCCATCCATCTATCA AGCTCCT	TCCTCCAG (#65)

2.3.3.5 Analysis of Taqman Quantitative PCR

The ABI HT Fast Real-Time PCR machines measure the fluorescence produced by the probes' Fam and Vic dyes over the course of the PCR reaction as the products are amplified. The fluorescence is displayed as an exponential curve against number of PCR cycles (an example is shown in Figure 2-2), with fluorescence directly representing the number of copies of the product present. For the gene of interest, a threshold was set within the exponential region of the fluorescence curves and the cycle number at which the plot for each sample reached the threshold was designated the Ct value for that well. The same process was repeated for the internal controls, *18S* or *GAPDH*. Ct values were collected for each of the triplicates and the Ct values for the gene of interest were standardised against the internal control Ct (gene of interest Ct minus internal control Ct) to give ΔCt. The ΔCt value accounts for variation in amount of mRNA in cDNA synthesis and differences in quantity of cDNA loaded between wells and samples. The ΔCt triplicates were averaged to give a mean value for each sample. For comparison between samples, the average ΔCt value of a designated control group was subtracted from the average ΔCt value of each sample, which gave the ΔΔCt value. Performing the transformation $y = 2^{-\Delta\Delta Ct}$ converts the ΔΔCt values to fold change (y) compared to the control group, which has a value of 1.

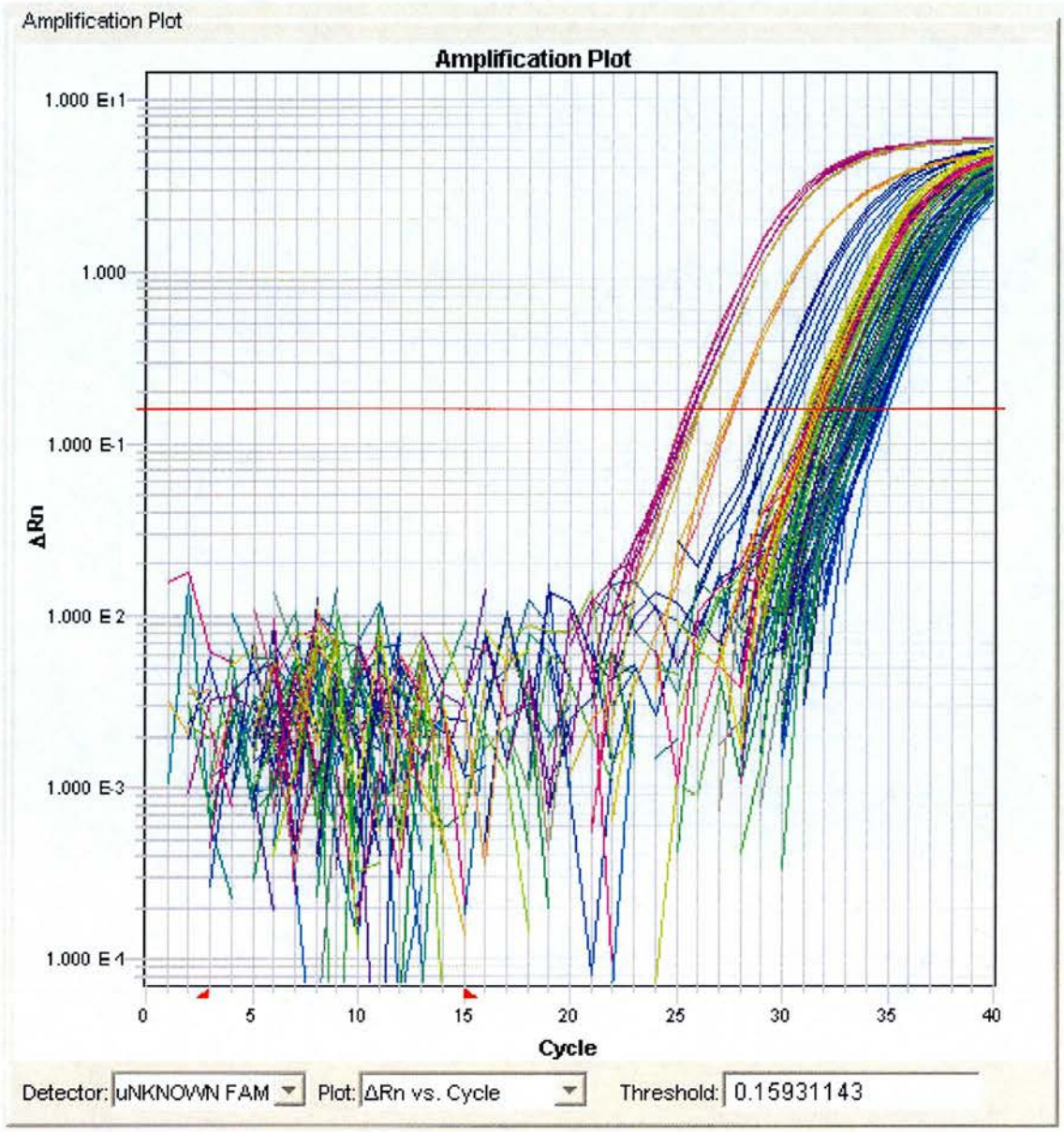


Figure 2-2: Example of TaqMan amplification plots for an unknown product labelled with Fam™. Horizontal line shows value set as 'threshold', note that it is within the region of exponential increase in fluorescence.

2.3.3.6 Validation of UPL primer/probe

Assay-on-demand primer/probe mixtures are validated by the supplier (Applied Biosystems) and therefore can be used without validation. The primers used in UPL

reactions are supplied independently of the probes and have not been validated for use in the assay by the supplier, therefore they need to be validated prior to use. Validation determines the efficiency of the PCR reaction using the primers and probe, and ensures that the gene of interest and endogenous internal loading control (18s) are amplified with the same efficiencies. Both these factors must be determined to allow calculation of fold-change and use of the ΔΔCt method. Both are determined by conducting real-time PCR in triplicate on 6 serially diluted aliquots (neat, 1/2, 1/4, 1/8, 1/16, 1/32) of a known positive sample using the primers and probe to be validated. To determine reaction efficiency average Ct on the Y-axis is plotted against log(sample concentration) on the X-axis, and the gradient of the line of best fit is calculated. A gradient of -3.32 and R^2>0.98 indicates a totally efficient reaction in which a 2-fold change in initial template concentration corresponds to a change in Ct value of 1. To determine that the templates of the endogenous control and gene of interest are amplified with the same efficiency the ΔCt value (gene of interest's Ct – endogenous control's Ct) on the Y-axis is plotted against log(sample concentration). A line of best-fit with gradient of approximately 0 (<0.1) indicates both templates are amplified with similar efficiencies.

2.4 Western Blot Analysis

2.4.1 Protein extraction and quantification

2.4.1.1 Total protein.

Protein was extracted from mouse and rat tissues and cultured SK11 cells using 1x RIPA buffer (Section 2.8.3). Frozen tissues were ground to powder in an ice-cold pestle and mortar under liquid nitrogen. Approximately 1 ml RIPA buffer was added per 0.3 g of ground tissue in a 1.5 ml Eppendorf™, and the tissue was homogenised with a handheld homogeniser (Sigma). Cells cultured in multi-welled plates were lysed directly in their wells using RIPA buffer and detached from the growth surface with cell scrappers, cells were incubated in buffer for 5 minutes on ice and then transferred to 1.5 ml Eppendorfs™. The volume of RIPA used to lyse cultured cells depended on well size, 6-well plates received 500 µl/well and 100 µl/well was added to 12-well plates. Once

samples from cells and tissue were lysed they were incubated on ice for 1 hour, and then spun at 6000 g for 10 minutes at 4°C in an Eppendorf™ centrifuge 5415R, to separate cell debris in the pellet from proteins in the supernatant. The extracted proteins were stored at -80°C prior to quantification and use.

2.4.1.2 Quantification of proteins

The protein concentration in samples was determined using DC Protein Assay (BioRad, Hemel Hempstead, Hertfordshire, UK) which is based upon the Folin-Lowry assay (Lowry et al., 1951). The assay was performed as prescribed in the protocol provided. Briefly, 5 µl of each sample were added in duplicate to a clear flat-bottomed 96-well plate. A series of BSA protein standards made up in 1x RIPA, with a range of concentrations from 0.125 to 1.5 mg/ml, were included in duplicate on the plate to provide a protein standard curve. To each well 25 µl of alkaline copper tartrate solution was added followed by 200 µl dilute Folin reagent, the solutions were mixed and incubated at room temperature for 15 minutes. Absorbance at 690 nm was measured in each well to quantify the colorimetric change, using a Labsystems Multiskan EX (Fisher Scientific UK Ltd, Loughborough, Leicestershire, UK). The absorbencies of the protein standards were plotted to give a standard curve from which the protein concentrations of the samples were determined.

2.4.2 Fluorescent LiCor Western blot

Pre-cast 4-12% graduated Bis-Tris mini NuPAGE gels (Invitrogen, Paisley, UK) were used to separate samples. Protein samples were added to NuPAGE loading buffer (section 2.8.3) and heated to 70°C for 10 minutes prior to being loaded. The NuPAGE gel was immersed in 1x MOPs NuPAGE SDS running buffer, which was prepared by diluting 50 ml 20x MOPs NuPAGE SDS running buffer (Invitrogen) in 950 ml dH_2O, plus 0.25% NuPAGE antioxidant (Invitrogen) for reduced samples. The gel was loaded with 20 µg/well of sample proteins and a protein ladder (NuPAGE SeeBlue ladder,

Invitrogen) and run at 200 V for 50 minutes. Following electrophoresis the gel was loaded into a transfer cassette in direct contact with HybondC super nitrocellulose (Amersham Life Sciences) or Immobilon-FL polyvinylidene fluoride (Millipore, Watford, UK) membrane with sponge and 3 mm Whatman papers (Scientific Laboratory Supplies Ltd., Nottingham) either side of the gel and membrane as shown in Figure 2-3. Protein transfer was performed overnight at 20 V or at 45 V for 3.5 hours in 20x NuPAGE Transfer buffer (Invitrogen) diluted 1 in 20 in dH_2O, 10% methanol and 0.1% NuPAGE antioxidant.

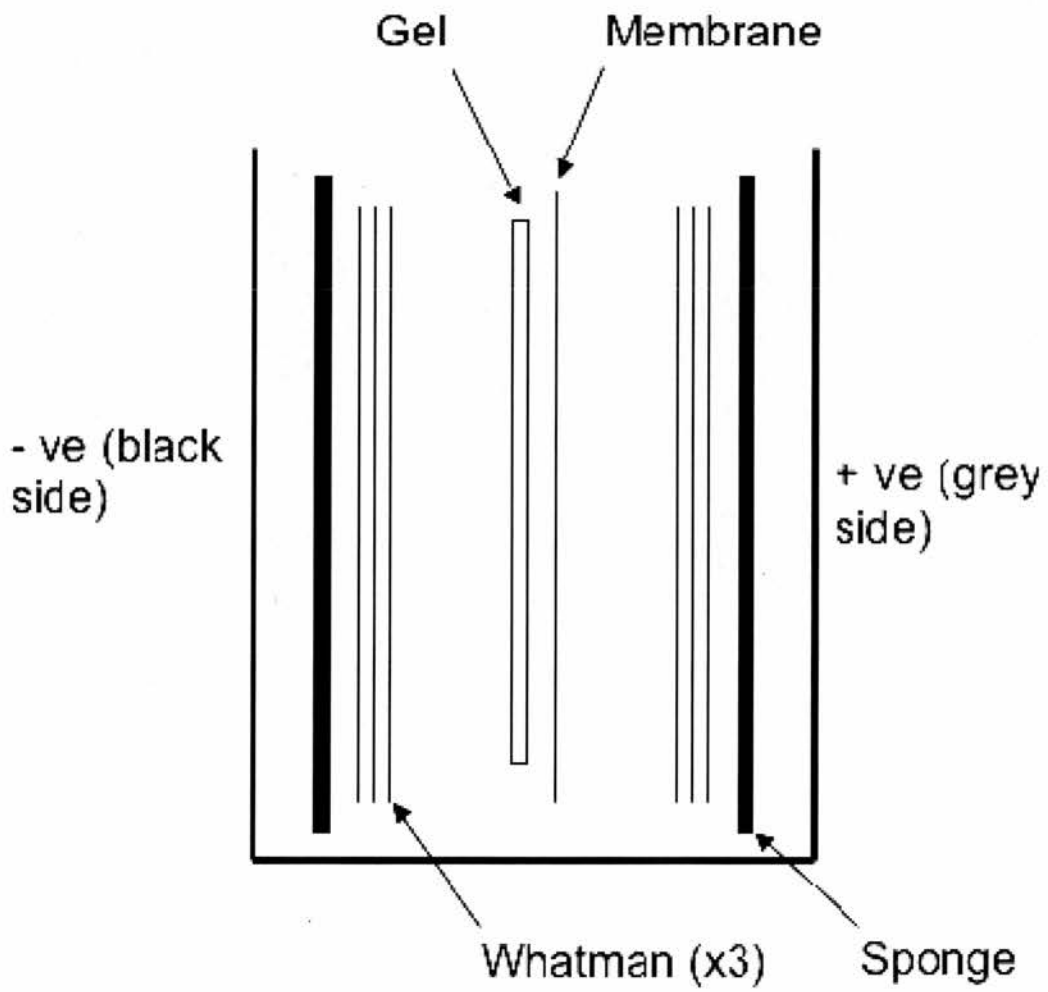


Figure 2-3: Orientation of components for transfer of protein from Bis-Tris gel to membrane in Western blotting.

Membranes were incubated at room temperature for 1 hour in Odyssey Blocking Buffer (LiCor Biosciences UK Ltd, Cambridge, UK), diluted 1:1 with PBS (phosphate buffered saline, Section 2.8.2), plus 20% goat serum. The blocked membranes were simultaneously exposed to two antibodies raised in different species and with specificity to either the protein of interest or the protein loading control (eg. β-actin), the antibodies were added to the blocking buffer plus 0.1% TWEEN-20 (Sigma) and incubated with the membrane for 1 hour at room temperature. The antibodies and dilutions used are shown in Table 2-4.

Table 2-4: Primary antibodies used for fluorescent (LiCor) Western blotting.

Antigen	Dilution	Species Raised	Source
AR	1:200	rabbit	US Biological
β-Tubulin	1:200	mouse	Sigma
β3-Tubulin	1:400	mouse	Sigma
Espin	1:5000	mouse	Transduction laboratoriess
Rhox5	1:1500	rabbit	Abcam
Sdmg-1	1:1000	rabbit	Gift from Ian Adams (MRC HGU, Edinburgh)
β-Actin (Loading control	1:5000	mouse	Sigma
β-Actin (Loading control)	1:1000	rabbit	Abcam

Secondary antibodies, raised against the species from which the primary antibodies were derived, were diluted 1: 10,000 in Odyssey Blocking Buffer (1:1 in PBS, plus 20% goat

serum, 0.1% TWEEN-20 and 0.01% SDS). Both secondary antibodies were labelled with fluorescent markers, the first emitting at a wavelength of 680 nm and the second at a wavelength of 800 nm. The fluorescent secondary antibodies used are shown in

Table 2-5.

Table 2-5: Fluorescent secondary antibodies used to visualise protein bands in Western blotting.

Target Organism	Raised In	Fluorescent label	Source
mouse	goat	Alexa fluor 680	Molecular Probes
rabbit	goat	IR dye 800	Rockland

By ensuring that the primary antibodies were derived from different species, each secondary antibody bound to only one primary antibody and therefore each protein was specifically labelled with fluorescence of a different wavelength. The position and intensity of binding for each secondary antibody and therefore the location and density of the protein bands were detected by scanning the membrane on the Odyssey scanner (LiCor Bioscience). Inclusion of the SeeBlue protein ladder indicated protein size. The band intensity was measured for the target protein and loading control, and intensity of the target protein was standardised to the loading control in each well. Comparisons of standardised intensities between samples were performed to determine any change in protein expression.

2.5 Immunohistochemistry

2.5.1 Tissue fixation, wax embedding, and sectioning

Tissues dissected from mice and rats for immunohistochemistry were immediately fixed in Bouins solution (Triangle Biomedical Sciences, Lancashire, UK) for 6 hours and then transferred to 70% ethanol until embedded in paraffin wax. Embedding was performed

over 24 hours on a Leica TP 1050 processor by the MRC Human Reproductive Sciences Unit histology support service. Tissue sections 5μm thick were cut from embedded tissues on a Leica RM 2135 microtome (Leica Microsystems UK Ltd, Milton Keynes, Buckinghamshire, UK) and floated in a warm (42°C) water bath. Individual sections were mounted on charged glass slides (BDH Superfrost® Plus, VWR international), dried at 65°C for several hours then at 50°C overnight. Sections to be immunostained were dewaxed by washing twice in xylene (Fisher Scientific UK Ltd) for 5 minutes and then rehydrated in alcohol baths of descending concentration (100%→100%→95%→70%) for 20 seconds in each bath, the sections were finally hydrated in tap water.

2.5.2 Detection with diaminobenzidene

2.5.2.1 Antigen retrieval

Antigen retrieval was performed with 0.01 M citrate buffer pH6. Sections were boiled in citrate buffer under full pressure for 5 minutes in a Tefal Clipso pressure cooker at the highest pressure setting, and were then rested for a further 20 minutes at atmospheric pressure without further heating. Sections were washed in an excess of cold tap water before peroxidase blocking.

2.5.2.2 Hydrogen peroxide block

Sections were blocked for 30 minutes with 3% hydrogen peroxide (BDH) in methanol and then washed once with tap water and three times with TBS, each TBS wash was 5 minutes.

2.5.2.3 Serum block

Sections were blocked in serum from the animal in which the secondary antibody to be used was raised. The blocking solution consisted of 1 part blocking serum (Diagnostics

Scotland)/4 parts TBS/5% BSA and was applied to each slide for 30 minutes at room temperature. Sections were washed 3 times with TBS, for 5 minutes each, following blocking with serum.

2.5.2.4 (Strep)Avidin-biotin block

For staining using antibodies and tissues where endogenous biotin caused non-specific background staining a (Strep)Avidin-Biotin block (Vector Laboratories Ltd., Peterbourgh, UK) was used. Neat avidin or strepavidin was applied to each section for 15 minutes at room temperature followed by three, 5 minute, washes in TBS. Neat biotin was added to each slide and incubated for 15 minutes at room temperature followed by a further three, 5 minute, washes in TBS.

2.5.2.5 Primary antibodies

Primary antibodies to target proteins were diluted in serum/TBS/BSA at appropriate dilutions (Table 2-6), and were incubated on sections overnight at 4°C. Thereafter sections were washed three times with TBS, for 5 minutes each. Primary antibodies were diluted in serum from the species in which the secondary antibody, intended for detection of the primary, was raised. Negative controls for which sections were incubated with serum/TBS/BSA, without primary antibodies, were included in each immunohistochemistry run. Sections in the negative control group were incubated with secondary antibodies in common with all other samples to reveal any non-specific binding of the secondary antibody. However pre-absorption of the primary antibodies with their peptide targets were not included as controls.

Table 2-6: Primary antibodies used in DAB immunohistochemistry.

Antigen	Dilution	Species raised	Source
AR	1:50	rabbit	Sigma
β-tubulin	1:1500	mouse	Sigma
Caspase-3	1:200	rabbit	Cell Signalling
SDMG-1	1:1000	rabbit	Gift from Ian Adams (MRC HGU, Edinburgh)
SGP-2	1:250	rabbit	Santa Cruz

2.5.2.6 Secondary antibodies

Sections were incubated with biotinylated secondary antibodies raised against the species from which the primary antibody was derived (Table 2-7). Secondary antibodies were diluted 1:500 in serum/TBS/BSA and incubated on sections for 30 minutes at room temperature. The serum in which the secondary antibody was diluted was from the same species in which the antibody was derived. After incubation slides were washed in TBS, 3 times for 5 minutes.

Table 2-7: Biotinylated secondary antibodies used in DAB immunohistochemistry.

Species against	Species Raised	Source
mouse	goat	DAKO (Ely, Cambridgeshire, UK)
rabbit	goat	DAKO

2.5.2.7 Labelling and visualising biotinylated secondary antibodies

The biotinylated label on the secondary antibody was further labelled either with ABC-HRP (DAKO) diluted in TBS (containing no salt) or Strepavidin-HRP diluted 1:1000 in

TBS (Figure 2-4), both were incubated with the sections for 30 minutes at room temperature. Slides were washed in TBS three times for 5 minutes and exposed to diaminobenzidene (DAB) to visualise the HRP label. DAB solution was prepared by diluting DAB substrate 1drop/ml in the supplied DAB buffer (DAKO).

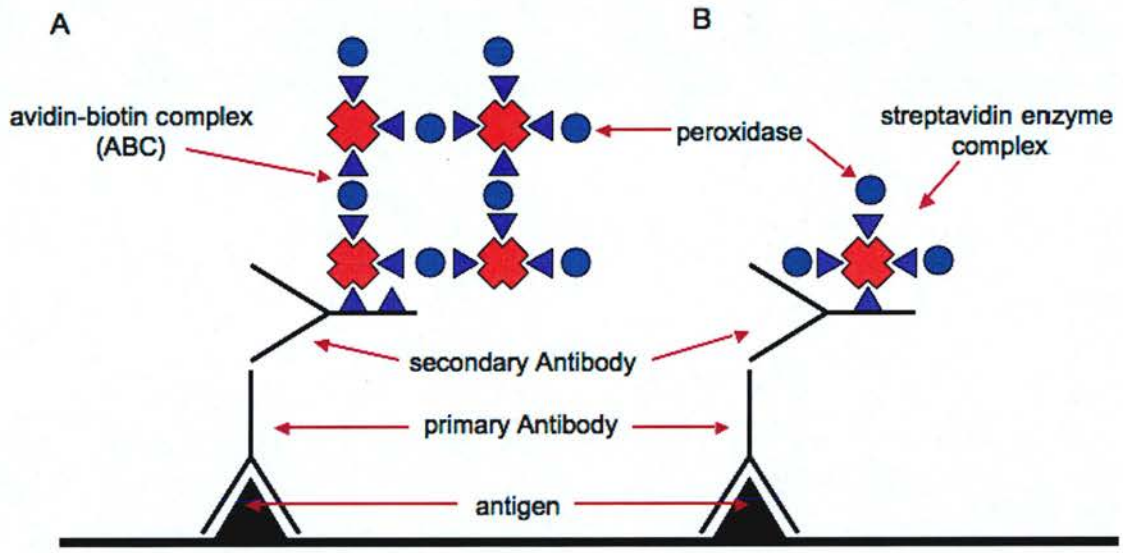


Figure 2-4: DAB staining using (A) avidin-biotin and (B) strepavidin enzymic complexes. Based upon figures published in Immunohistochemical Staining Methods, Fourth Edition, Editor Marc Key (Key, 2006).

2.5.2.8 Counter staining and mounting

DAB stained sections were counter stained in haemoxylin for 5 minutes. Washing the sections in acid-alcohol, for 5-20 seconds, removed the haemoxylin from the cytoplasm of cells more rapidly than from the nucleus resulting in blue nuclear staining when developed in Scott's tap water, for 30 seconds. Slides were dehydrated in graded alcohol washes (70%→80%→95%→100%→100%) for 20 seconds each, and then 2 xylene washes of 5 minutes. The slides were coverslip mounted using Pertex mounting media (CellPath, Hemel Hempstead, UK).

2.5.2.9 Bond-X automated immunohistochemistry

Sections immunostained using the Bond-X machine (Leica, Newcastle Upon Tyne, UK) were prepared as described above up to and including citrate retrieval. The sections were loaded onto the machine and the automated immunostaining assay procedure was performed. The appropriate primary antibodies (Table 2-8) were diluted in Bond™ primary antibody diluent (Leica) and incubated on the sections for 1 hour during the course of the protocol. The automated process includes all steps up to and including counter staining, so upon completion of the run sections were dehydrated and mounted with Pertex (Section 2.5.2.8)

Table 2-8: Primary antibodies used for automated immunohistochemistry using Bond-X machine.

Antigen	Dilution	Animal raised	Source
GFP	1:1000	rabbit	Molecular probes (Invitrogen)
HIF-1α	1:200	rabbit	Abcam

2.5.3 Fluorescence

Sections for fluorescent staining were prepared for incubation with primary antibodies as for DAB staining (Sections 2.5.1 to 2.5.2.4), except for the use of PBS in place of TBS. The hydrogen peroxidase-blocking step was only included when a peroxidase tagged secondary antibody was to be used. Primary antibodies used in fluorescent immunohistochemistry are shown in Table 2-9 and were diluted in serum/PBS/BSA at the dilutions shown, the serum used was from the species in which the secondary antibody to be used was derived.

Table 2-9: Primary antibodies used in fluorescent immunohistochemistry.

Antigen	Dilution	Raised in	Source
CD 68	1:50	mouse	DAKO
Connexin43	1:100	rabbit	Zymed
Espin	1:30	mouse	Transduction Laboratories
N-cadherin	1:7000	mouse	Zymed
SMA	1:500	mouse	Sigma
MPO	Pre-diluted	rabbit	Abcam
ZO-1	1:100	rabbit	Zymed

2.5.3.1 Fluorescent labelling of primary antibodies

The fundamental difference between DAB and fluorescent immunohistochemistry was the use of secondary and/or tertiary antibodies tagged with fluorescent labels to detect bound primary antibodies; the antibodies used are listed in Table 2-10. Often a secondary antibody directly conjugated to the fluorescent tag was used (panel A, Figure 2-5). To achieve greater sensitivity for low abundance targets a biotin- or peroxidase-tagged secondary antibody was used and then fluorescently labelled in a further step: biotin-tagged secondary antibodies were detected with a tertiary antibody with a fluorescent label (panel B, Figure 2-5), peroxidase labelled antibodies were fluorescently tagged with fluorescyl-tyramide (panel C, Figure 2-5). In double fluorescent immunohistochemistry 2 primary antibodies raised in different species and targeting different antigens were used, and were detected with 2 combinations of secondary and/or tertiary antibodies, labelling primary antibodies of each species with different fluorescent markers.

Detection using two primary antibodies raised in the same species (mouse) was achieved using FAB secondary antibodies labelled with biotin, and two tertiary complexes (avidin and strepavidin) labelled with different fluorescent labels (panel D, Figure 2-5). The FAB antibodies are monovalent, meaning they lack one of the two antigen-binding domains found on conventional secondary antibodies (dotted line, panel D, Figure 2-5). Therefore when the FAB antibody binds to a primary antibody all its binding sites are occupied. A second primary antibody raised in the same species as the first can be added to the same section without the FAB antibody capturing the new primary antibody and interfering with staining, as would occur with a conventional secondary antibody (Negoescu et al., 1994). Antibodies are added in the order described above: first primary antibody followed by FAB secondary antibody, then the second primary antibody followed by the FAB secondary antibody.

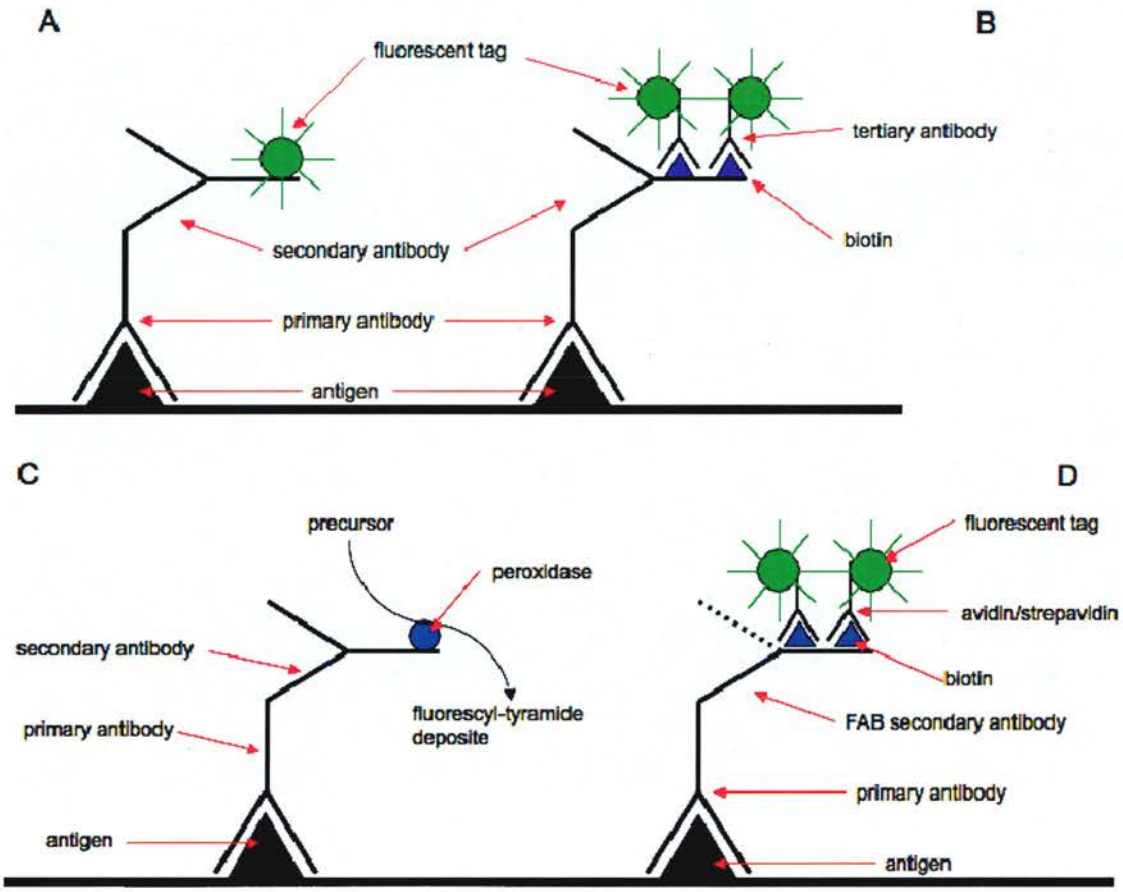


Figure 2-5: Methods for visualising primary antibody binding in fluorescent immunohistochemistry. Fluorescent staining using: a secondary antibody directly conjugated to fluorescent tag (A), a tagged secondary antibody and fluorescently labelled tertiary complex (B), a secondary antibody tagged with peroxidase catalysing fluorescyl-tyramide deposition (C), and FAB secondary antibodies and fluorescently labelled tertiary complexes (D).

For fluorescent immunostaining PBS was used in place of TBS to wash the slides and dilute antibodies and reagents. In wash steps prior to use of a fluorescent secondary antibody PBS alone was used, but in all washing steps following incubation with a fluorescent secondary antibody PDS plus 0.05% TWEEN-20 was used for the first of the 3 washes. The sections were counterstained with the nuclear stain DAPI (Sigma),

diluted 1:1000 in PBS for 10 minutes. Sections were mounted under coverslips using Permafluor (Beckman Coulter, High Wycombe, UK).

Table 2-10: Secondary and tertiary antibodies used in fluorescent immunohistochemistry.

Species against	Species raised in	Label	Dilution	Secondary /tertiary	Source
mouse	goat	peroxidase	1:200	secondary	DAKO
-	-	Tyr Fluorescein	1:50	tertiary	PerkinElmer LAS (UK) Ltd (Beaconsfield, Buckinghamshire, UK)
rabbit	goat	biotin	1:500	secondary	DAKO
-	-	Strepavidin Alexa 546	1:200	tertiary	Molecular probes
mouse	goat	Alexa488	1:200	secondary	Molecular probes
mouse	goat	Alexa546	1:1000	secondary	Molecular probes
-	-	Avidin Alexa 488	1:200	tertiary	Molecular Probes
mouse	goat	Biotinylated FAB	1:500	secondary	Abcam

2.5.4 Imaging

2.5.4.1 Light microscopy

DAB immunostaining was visualised on an Olympus Provis microscope (Olympus Optical Co.) and images were captured with a digital Canon EOS 30D camera (Canon Europe, Amsterdam).

2.5.4.2 Meta-confocal microscopy

Tissues and cells stained in fluorescent immunohistochemistry were observed on a Zeiss LSM 510 laser scanning meta confocal microscope (Carl Zeiss Ltd, Welwyn Garden City, UK). Green fluorescence was excited with the Argon2 488 nm laser, red fluorescence by the HeNe1 543 nm laser, and blue (DAPI) fluorescence with the laser Diode 405 nm. Fluorescence at each wavelength was recorded separately, and images are a composite of all channels.

2.6 Adenoviral construct amplification

The following procedures were carried out by staff in the MRC Human Reproductive Sciences Unit's Biomolecular Core Facility (managed by Dr. Pamela Brown), to whom I am very grateful.

Four adenoviral constructs were used in the course of these studies, the construct used in chapter 3 contained *ERE-Tk-luc* and the constructs used in chapter 4 expressed *green-fluorescent protein* (*GFP*), *LacZ*, or contained no insert. The sources and methods of preparation of the constructs are described in the appropriate chapters, however the high titre virus stocks required were generated using the following method in common.

The adenoviral constructs were multiplied *in vitro* by infection of HEK293 (Graham et al., 1977) cells at 5-7 viral particles per cell (known as the multiplicity of infection or MOI). The HEK293 cells were maintained in MEM media plus 10% FBS and penicillin-streptomycin at 37°C in 5% CO_2 until the cells showed a cytopathic response; virus was

released from the cells by 3 cycles of freeze thawing. The viral particles were purified by buffer exchange with 8 volumes of 2.5% glycerol, 20mM Tris.HCl pH8 using a Vivascience AdenoPack column (Generon House, Eton Wick, UK) and then concentrated. The viral titre, in plaque forming units (pfu)/ml, was quantified on HEK293 cells using an Adeno-X rapid titre kit (BD Bioscience, Oxford, UK) and 1:500 rabbit anti-adenovirus serotype 5 hexon antisera (Autogen Bioclear UL Ltd, Calne, UK) to label infected cells.

2.7 Statistical Analyses

All statistical analyses were conducted using GraphPad Prism 5, in all analyses a confidence level of $p \leq 0.05$ was deemed significant. Two-way analysis of variance (ANOVA) was used to determine statistical significance when samples underwent two independent treatments, for example in studies where cells were cultured at 34°C or 39°C and treated with steroids at a range of concentrations. Although results were presented standardised against a control sample and are based upon small numbers of repeats (n=2 or 3) indicating use of a non-parametric test, no non-parametric test with capacity to analyse the studies as designed exists so the two-way ANOVA was chosen. In two-way ANOVAs where significant differences were reported, the treatments between which statistical significances were found were identified using the Bonferoni post-hoc test. A Mann-Whitney analysis was used for comparisons in studies where samples underwent one of two treatments, i.e. cells cultured at 34°C or 39°C, and small n-numbers (n=3 experiments) were present in each group making the parametric t-test inappropriate. The third statistical analysis used was the non-parametric Kruskal-Wallis test, this analysis was used to analyse data in which more than two treatments of the same type were used, for example when analysing expression of mRNA or protein in control, EDS and EDS plus TE treated rats. The non-parametric test was used when n-numbers were small or expression data were presented standardised to a control, valued as 1, because these conditions prohibit use of the parametric one-way ANOVA. Significant results in the Kruskal Wallis test were investigated further using Dunn's

multiple comparison post-hoc test to identify the treatments between which significant differences lay.

2.8 Commonly used solutions

2.8.1 Tissue culture

'Complete' medium: Dulbecco's Modified Eagles Medium (DMEM, Sigma), supplemented with:
10% (v/v) heat-inactivated foetal bovine serum (FBS, Gibco, Paisley, UK)
100 U/ml Penicillin & 100µg/ml Streptomycin (Gibco)
1% (v/v) Non-essential amino acids (Sigma) 125 ng/ml Fungizone (Gibco)
1% (v/v) D(+) Glucose (45% solution, Sigma)
1% (v/v) 2 mM L-Glutamine (Gibco)
5 µg/ml Plasmocin (Autogen Bioclear, Wiltshire, UK)

Prior to experimental treatments; such as dosing with ligands, culture at altered temperatures, and transfection/infection; the cells were cultured in 'complete' media in phenol-red free DMEM (Sigma) with charcoal-stripped foetal bovine serum.

Transfection medium: Phenol-red free DMEM (Sigma), supplemented with:
1% (v/v) Non-essential amino acids (Sigma)
1% (v/v) D(+) Glucose (45% solution, Sigma)
1% (v/v) 2 mM L-Glutamine (Gibco)

Freezing medium 90% 'Complete' medium
10% filter sterilised DMSO

2.8.2 Immunohistochemistry

1xTBS	Tris (Sigma)	60.5 g
	Sodium Chloride (Sigma)	87.6 g
	Hydrochloric acid (BDH)	300 ml
	Adjust to pH 7.4 with Conc. Hydrochloric acid	
1xPBS	NaCl	8 g
	KCl	0.2 g
	Na_2HPO_4	2.29 g
	KH_2PO_2	0.2 g
	Make up to 1 litre in dH_2O	

2.8.3 Molecular Biology

50x TAE	242 g Tris (Sigma)
	100 ml 0.5 M EDTA (Sigma)
	57.1 ml acetic acid (BDH)
	made up to 1 litre with dH_2O

1x RIPA buffer	1% Triton X-100 (Sigma) 15 mM HEPES-NaOH (pH 7.5) (Sigma) 0.15 mM NaCl 1% Sodium deoxycholate (Sigma) 0.1% Sodium dodecyl sulfate (SDS) (Sigma) 1 mM Sodium orthovanadate (Sigma) 10 mM EDTA (Sigma) 0.5% Protease inhibitors cocktail (Roche) Protease inhibitors were added on the day of use.
NuPAGE Loading buffer	20 µg Protein 2.5 µl NuPAGE LDS Sample Buffer (4x) 1 µl NuPAGE Reducing Agent (10x) made up to 10 µl with dH_2O.

3 Gene expression studies using an immortalised Sertoli cell line

3.1 Introduction

Sertoli cells support development of the germ cells associated with them. This support is provided in the form of factors secreted by the SC as well as physical interactions between SC and GC. Physical interactions include junctions such as ectoplasmic specialisations, gap junctions, tubulobulbar complexes, and desmosome-like junctions (McGinley et al., 1979; Russell, 1977a; Russell, 1977b; Russell and Clermont, 1976); SC-secreted factors that influence GC development include activin, steel factor and GDNF (Marziali et al., 1993; Matsui et al., 1991; Meehan et al., 2000; Meng et al., 2000)(as described in section 1.2.3). Recent studies in mice including those in which a SC-specific knockout of AR expression has been achieved demonstrate the importance of somatic cell function as these mice are infertile because germ cells fail to complete meiosis efficiently (De Gendt et al., 2004). Oestrogen signalling also plays a role in maintaining male fertility. In mice lacking a functional aromatase (*Cyp19*) gene GC apoptosis increases, abnormal acrosome development occurs, and spermatids detach from the seminiferous tubules (Robertson et al., 1999). ERß is expressed in both somatic and germ cells in rodents and primates (Saunders et al., 1997; Saunders et al., 2001; van Pelt et al., 1999; Zhou et al., 2002) and ERα has been detected in murine LC and PTM with highest levels of expression in the efferent ductules (Fisher et al., 1997; Hess et al., 1997b; Pelletier et al., 2000; Zhou et al., 2002). Interference with oestrogen signalling by knocking out ERβ also causes male infertility, although the mechanism has not been identified it may be behavioural as no abnormalities in testis and epididymis histology or spermatozoa motility have been observed (Antal et al., 2008).

As described in section 1.3.2.1 steroid hormone receptor activation results in dimerisation of the receptor and recruitment of transcriptional cofactors to initiate expression of responsive genes via hormone response elements (HRE) in their promoter

regions (Beato et al., 1989; Doesburg et al., 1997; Ikonen et al., 1997; Wong et al., 1993). As androgen-dependent signalling in SC is essential for complete spermatogenesis, identification of androgen responsive genes in SC has been undertaken. Notably the expression of AR itself is androgen responsive; AR expression in SC is dependent on high intratesticular testicular testosterone (Bremner et al., 1994; Turner et al., 2001). The homeobox gene, *Rhox5*, which is expressed in SC and the epididymis is also androgen-responsive (Lindsey and Wilkinson, 1996a; Lindsey and Wilkinson, 1996b). Two promoter regions (distal and proximal) have been identified for *Rhox5*; the proximal promoter contains two androgen response elements and is responsible for expression within the SC (Barbulescu et al., 2001; Sutton et al., 1998). Further studies to investigate differential gene expression between SC with or without androgen receptor expression have identified numerous putative androgen responsive genes including *β3-tubulin*, *claudin3*, *claudin11* and *espin* (Abel et al., 2008; Denolet et al., 2006a; Meng et al., 2005).

Sertoli cells isolated from the testes of rodents between the ages of 18 and 21 days have been used to investigate the functions of these cells in the relatively convenient setting of *in vitro* cultures, and a method for isolation has been described by Karl and Griswold (Karl and Griswold, 1990). However in addition to being rapidly outgrown by the peritubular myoid cells that contaminate the SC cultures (Karl and Griswold, 1990; Schlatt et al., 1996) the phenotype of the SC is unstable and there are reports of dedifferentiation of the cells within only a few days. For example, expression of AR is lost rapidly in cultured primary SC; after 3 days in culture AR expression is still detected but soon declines (Denolet et al., 2006b; Nakhla et al., 1984). In SC-enriched cultures derived from rat tissue and maintained over the course of 6 or 16 days, secretion of androgen-binding protein (ABP) can be used as a marker of SC function. The secretion of ABP declined dramatically in cells in the absence of hormones over the culture period (reduced by 6-8 days), although treatment with hormones including testosterone, insulin and FSH increased ABP secretion above that in control cells (Karl and Griswold, 1980; Rommerts et al., 1978). In cultures of primary rat SC, proliferation can be maintained

beyond 22 days in culture by FSH treatment. However by 26-32 days of culture the SC develop an abnormal phenotype characterised by cytoplasmic vacuoles, increased cytoplasmic area and multiple nuclei per cell (Buzzard et al., 2002).

As described in chapter 1, section 1.5.1, immortalised cells have been used in place of primary SC in an attempt to study SC in an *in vitro* setting whilst avoiding the problems described above, such as dedifferentiation and loss of steroid hormone receptor expression. Section 1.5.1 describes three immortalised SC cell lines: Tm4 (Mather, 1980), MSC-1 (Peschon et al., 1992), and SK11 (Walther et al., 1996); it is the last of these that was utilised for the studies presented in this chapter. The SK11 cell line is derived from SC isolated from the testes of a ten-day-old *H-2K^{b}-tsA58* mouse, these mice express the *tsA58* large T-antigen in cells throughout their body and therefore in all cells derived into culture (Jat et al., 1991), as described in section 1.5.2. The temperature-sensitive *tsA58* is expressed when SK11 cells are cultured at permissive temperatures (i.e. those below 39°C), e.g. at 34°C used in this study permitting proliferation in an undifferentiated state (Walther et al., 1996). When SK11 cells are cultured above the permissive temperature for at least 48 hours the large T-antigen is inactivated and proliferation ceases, the phenotype of the cells also changes towards that of a differentiated SC (Jat et al., 1991). The expression of various genes at permissive and non-permissive temperatures were described in section 1.5.2, it is relevant to this chapter to repeat that whilst the expression of *SGP-1* is similar at permissive and non-permissive temperatures, expression of *SGP-2* is raised in differentiated cells at non-permissive temperatures (Sneddon et al., 2005; Walther et al., 1996; Walther et al., 1997), which suggests differentiation at the higher temperature.

Androgen and oestrogen receptors, AR and ERβ, are expressed by mouse SC *in vivo* (Suarez-Quian et al., 1996; Zhou et al., 2002), and both have been detected in the SK11 cell line (Sneddon et al., 2005; Walther et al., 1996). ERα has not been detected in SC *in vivo* (Zhou et al., 2002) and no mRNA was expressed in SK11 cells (Sneddon et al., 2005). There was no detectable difference in expression of ERß in SK11 cells cultured at

34°C and 39 °C, however *AR* mRNA and protein expression were increased at 39°C (Sneddon et al., 2005). Expression of steroid hormone receptors in SK11 cells strongly suggests that these cells retain androgen and oestrogen responsiveness. In a previous study from our laboratory treatment of SK11 cells with testosterone or DHT, but not oestradiol or 3βAdiol, stimulated a reporter construct linked to the androgen-responsive *Rhox5* promoter demonstrating expression of a functional AR protein in the cells (Sneddon et al., 2005). Stimulation of a reporter construct under control of three tandem EREs was achieved in SK11 cells by treatment with oestradiol or 3βAdiol, proving the presence of functional ERβ (Sneddon et al., 2005). The same study also reported that expression of mRNA for *AR* and *ERβ* are increased by androgen and oestrogen treatment respectively (Sneddon et al., 2005).

3.1.1 Aims

The aims of the studies presented in the following chapter were to extend the investigation of androgen and oestrogen response in SK11 cells, and to identify genes differentially expressed in the cells under control of these steroids. By using SK11 cells as a model of *in vivo* SC action it was hoped that genes involved in control of spermatogenesis by action of androgens and oestrogens on SC would be identified and their roles could be investigated.

3.2 Materials and methods

3.2.1 Cell culture

SK11 cells were incubated at 34°C in 5% CO_2 in 'complete' culture media and passaged every 2-3 days to maintain a stock of proliferating (undifferentiated) cells (section 2.2.2). The cells were cultured in phenol red-free 'complete' media (section 2.8.1) for 48 hours prior to and during steroid treatment, and for 48 hours prior to RNA/protein extraction in proliferative versus differentiated cell studies. To differentiate cells they were cultured for 48 hours at 39°C and then incubated as described below if further treatments were required. The cells were used to provide materials for RT-PCR and Western blot analysis (sections 2.3.1 and 2.4.1).

3.2.1.1 Steroid treatments

Both proliferating and differentiated SK11 cells were treated with androgens (testosterone, DHT) or oestrogens (oestradiol (E2). diethylstibestrol (DES), 3βAdiol) at doses between $1x10^{-12}$ and $1x10^{-6}$ M. Stocks of these steroids were prepared as described in section 2.2.3. Control cells were incubated with appropriate vehicle of ethanol or DMSO (1:100 in PBS) diluted 1:1000 in growth media. In studies with oestrogens the oestrogen receptor antagonist ICI 182,780 (1μM) was added to control groups 1 hour prior to oestrogen treatment and was also included with some steroid treatments to demonstrate oestrogen receptor specificity of the effects.

3.2.2 Transient transfection

The plasmids used in transient transfections were mouse *AR* (*mAR*), the proximal *Rhox5* promoter linked to the firefly (*Photinus pyralis*) *luciferase* (*Rhox5-luc*), and *Renilla reniformis luciferase*. Expression of the *mAR* plasmid is under control of the cmv5Tb promoter and the *Rhox5* promoter contains 2 androgen response elements whose sequences are: AGATCTcattcTGTTCC (ARE-1) and AGCACAtcgTGCTCA (ARE-2). The *mAR* plasmid was a kind gift from Dr. Frank Claesans (Leuven, Belgium), the *Rhox5-luc* plasmid was a gift of Professor G. Verhoeven (Leuven, Belgium), and the

Renilla plasmid was supplied by the MRC Human Reproductive Sciences Unit's Biomolecular Core Facility (managed by Dr. Pamela Brown).

SK11 cells were seeded onto 6-well plates at $4x10^5$ cells/well, in phenol-red free 'complete' medium, 48 hours prior to transfection and cultured at 34°C. The 'complete' media was removed from the cells 1 hour before transfection and replaced with 500 µl 'transfection' medium (section 2.8.1). Plasmids (*mouse AR*, *Rhox5-Luc*, and *Renilla*) were transfected into cells using JetPEI (Qbiogene, Cambridge, UK), as shown in Figure 3-1. Briefly, plasmids were diluted in 50 µl NaCl (50 mM) per well, and JetPEI was diluted in a separate 50 µl NaCl per well. The JetPEI solution was added to the plasmid solution, mixed gently by pipetting, and incubated at room temperature for 30 minutes. JetPEI/DNA solution (100 µl/well) was added to the transfection medium in each well drop wise and mixed by swirling gently. The cells were incubated with the transfection reagent and DNA for 4 hours at 37°C in 5% CO_2 after which the transfection reagents was removed and replaced with 2 ml transfection medium plus 10% carbon-stripped FBS, supplemented with DHT or DMSO when required. Cells were returned to 34°C in 5% CO_2 for 6-48 hours and expression of mRNAs (*AR*, *Rhox5*, *Cldn3*, and *Cldn11*) or proteins (AR or luciferase) were assessed (sections 3.2.4.2 and 3.2.2.1 respectively).

SK11 cells were either transfected with 300-1500 ng *mAR* per well or with 500 ng *mAR*, plus 2.5 µg *Rhox5-luc* and 625 µg *Renilla* per well. Depending upon the plasmids being introduced into the cells the quantity of DNA being transfected ranged from 300 ng to 3.625 µg/well. Transfection using JetPEI requires an optimal ratio between the nitrogen in the JetPEI and the phosphates in the plasmids, called the N/P ratio, which is 5 for SK11 cells. The following equation determines the volume of JetPEI used in the transfection mixture for a N/P ratio of 5:

$$\text{µl of JetPEI to be used} = \frac{(\text{µg DNA} \times 3) \times \text{N/P ratio}}{7.5}$$

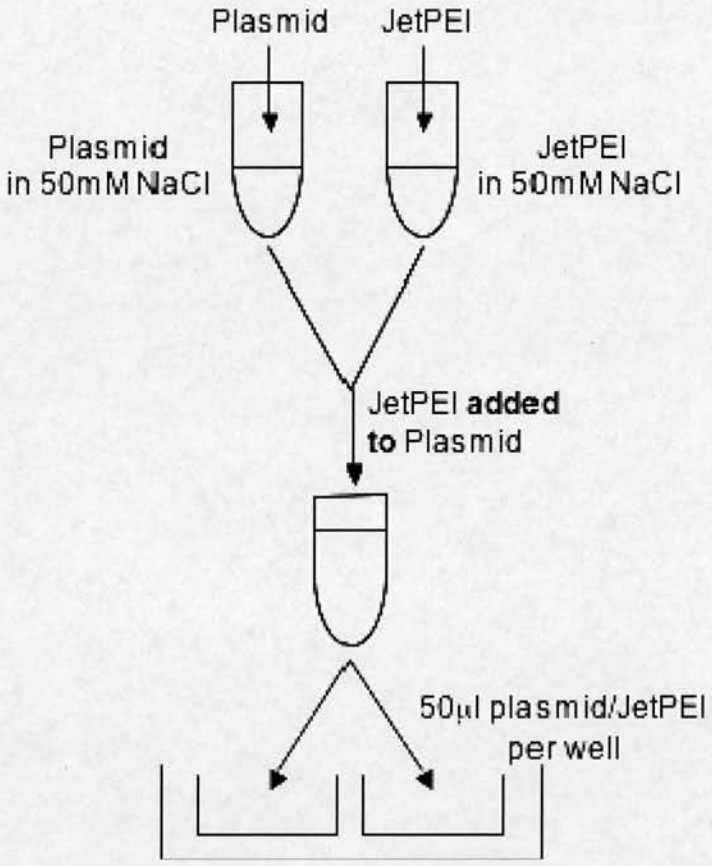


Figure 3-1: Scheme for transient transfection of SK11 cells using JetPEI.

3.2.2.1 Luciferase assay.

Expression of firefly and renilla luciferase by SK11 cells following transfection with the *Rhox5-luc* and *Renilla* plasmids was quantified using the Promega Dual Luciferase Reporter™ assay. The assay was performed in a 96-well plate as described in the manufacturer's protocol, and luminescence was measured on a Fluostar optima luminometer (BMG labtech, Aylesbury, UK). Briefly, the protocol involves lysis of the cells with 150 µl 1x passive lysis buffer for 15 minutes at room temperature. Twenty microlitres of cell lysate/sample was pipetted onto a 96-well plate and the lysates were assayed using an automated protocol on the luminometer. The luminometer introduces 100 µl of luciferase assay reagent II (LARII) into a well and measures the luminescence

due to the presence of firefly luciferase. After 8 seconds the luminometer introduces 100 µl of Stop & Glo® reagent to quench firefly luciferase luminescence and activate renilla luciferase luminescence, which is measure for 7 seconds. The process is repeated for each well in turn. The average luminescence due to firefly luciferase is standardised to average renilla luminescence, to normalise for differences in transfection efficiency and number of cells present.

3.2.3 Adenoviral ERE-Tk-luc construct

The *ERE-Tk-Luc* construct was a kind gift of S.C. Nagel and D.P. McDonnell (Duke University, USA). The construct consists of 3 copies of the *vitellogenin ERE* linked to the *TATA-Luc* reporter (Hall and McDonnell, 1999). The construct was introduced into an adenoviral construct as described below. The sequence of the promoter region of the reporter construct is shown below, the locations of the 3 ERE repeats are underlined:
TAGGTCAcagTGACCTGCGGATCCGCAGGTCActgTGACCTAGATCCGCAGGTC
ActgTGACCT

3.2.3.1 Preparation of construct

The following procedures were carried out by staff in the MRC Human Reproductive Sciences Unit's Biomolecular Core Facility (managed by Dr. Pamela Brown), to whom I am very grateful.

The *ERE-Tk-Luc* plasmid was subcloned into the *pDC316* shuttle plasmid, which contains a loxP recombination site. The shuttle plasmid, bearing the *ERE-Tk-Luc*, was co-transfected with the adenoviral genomic plasmid *pBHGloxΔE1,3Cre* in HEK293 cells. The adenoviral genomic plasmid lacked both the *E1* and *E3* regions rendering the genome replication incompetent in the absence of a helper cell line such as HEK293, and a Cre and loxP site for incorporation of the shuttle. Co-transfection in the HEK293 cells yielded a recombinant viral vector containing the *ERE-Tk-Luc* in place of the *E1* domain and also lacking the *E3* region. The plasmids and reagents were supplied by Microbix Biosystems Inc. (Toronto, Ontario, Canada), and the procedure was as

described in their protocol. High titre stocks of the *ERE-Tk-luc* construct were generated by amplification in HEK293 cells as described in Section 2.6.

3.2.3.2 Viral infection

Prior to infection with the adenoviral ERE, SK11 cells were cultured in 96-well plates at 7.5 x10^3 cell/well for 48 hours at 39°C in phenol red-free 'complete' media. Media was removed from each well and replaced with 400 µl/well of fresh media. The *ERE-luc* viral stock was diluted in phenol red-free 'complete' media to a concentration of 50 pfu/cell (50 MOI) in 50 µl/well. Each well was infected with 50 µl of diluted viral solution and incubated for 4 hours at 37°C in 5% CO_2. The media and virus were removed and replaced with phenol red-free media and cells were returned to culture at 39°C. The cells infected with *ERE-luc* were incubated with oestrogen ligands (section 3.2.1.1) for between 12 and 48 hours, in 5% CO_2, at 39°C.

3.2.3.3 ERE-luciferase assay

SK11 cells infected with the *ERE-Luc* adenovirus were treated with E2, DES, or 3βAdiol at concentrations from 10^{-12} to 10^{-7} M, plus or minus 1 µM ICI 182,780. Cells in control wells were treated with 1 µM ICI 182,780 (section 3.2.1.1).

Two distinct studies were undertaken. The first was a time course study in which *ERE-Luc* infected cells were treated with E2 (10^{-8} or 10^{-7} M) plus and minus ICI 182,780 or DES (10^{-8} M) for 12, 24, 36 and 48 hours. At each time point luciferase protein was extracted from the cells for quantification (section 3.2.3.4).

In the second study SK11 cells were exposed to E2 (10^{-11} - 10^{-7} M), DES (10^{-12} - 10^{-7} M), and 3βAdiol (10^{-11} - 10^{-7} M). A duplicate of each treatment was also performed with ICI 182,780 (1 µM) added 1 hour prior to and throughout ligand treatment. Cells were exposed to the treatment for 24 hours before the luciferase was extracted and analysed. The luciferase assay was performed as described in section 3.2.3.4.

3.2.3.4 Bright-glo Luciferase assay

Luciferase expression was quantified using the BrightGlo luciferase assay (Promega) in accordance with the provided protocol. SK11 cells were lysed in 100 µl of glo lysis buffer per well for 5 minutes at room temperature, and 90 µl/well of the lysed solution was transferred to a white polystyrene 96-well assay plate (Fisher Scientific). An equal amount (90 µl) of BrightGlo reagent was added to each well and the solutions were incubated together for 2 minutes before luminescence was measured on a Fluostar optima luminometer.

3.2.4 PCR

3.2.4.1 Semi-quantiative RT-PCR

RNA extracted from SK11 cells and adult mouse testis was used to synthesis cDNA in an oligo-dT primed reaction as described in section 2.3.1. A standard PCR reaction catalysed by Taq polymerase (Bioline) was prepared using primers to *AR*, *Rhox5*, *SGP-1*, and *SGP-2*. Primer sequences, predicted product size and annealing temperatures for each reaction are shown in Table 3-1.

Table 3-1: RT-PCR primer sequences, product size, and annealing temperatures

Gene	5' Primer Sequence	3' Primer Sequence	Product Size (bp)	Annealing Temperature (°C)
AR	GGACCATGTTTTAC CCATCG	TCGTTTTCTGCTGG CACATAG	171	56
ERβ	CCAATGTGCTAGTG AGCCG	AACTCACGGAACCG TGCCG	393	55
Nested *ERβ*	TGCTAGTGAGCCGT CC	CCAAAGATTTCCAG AATCC	348	50
GAPDH	CTGCACCACCAACT GCTTAGC	ATGCCAGTGAGCTT CCGTTC	288	58
Rhox5	GTGGACAAGAGGA AGCACAA	TCTCCCCATCTCAC TCCAC	635	55
SGP-1	TAAGGCTAACGAG GACGTCTGC	GCCTGGACCAGATT CTGCTCAT	484	58
SGP-2	CATCTGGCATCATA GACACGCT	ACACAGTGCGGTCA TCTTCACC	460	58

3.2.4.2 TaqMan Quantitative RT-PCR

Samples of RNA extracted from mouse tissues (testis, prostate, epididymis and kidney) and SK11 cells were incubated in a random hexamer primed reverse transcriptase reaction to produce cDNA (section 2.3.3.1). The cDNA was used for TaqMan in both Assay-On-Demand™ (section 2.3.3.3) and the Universal Human Probe Library™ reactions (section 2.3.3.4) undertaken on an ABI 7900 HT Real-Time PCR Machine.

Details of the Assay-On-Demand reagents are shown in Table 3-2, the sequences of the primers and probes in the UPL TaqMan are shown in Table 3-3.

Table 3-2: Taqman Assay-On-Demand™ primer/probe assays for TaqMan.

Gene	Assay-On-Demand Cat. No.
AR	Mm00442688_m1
Rhox5	Mm00476718_m1
SGP-2	Mm00442771_m1

Table 3-3: Universal Human Probe Library™ primer and probe sequences for TaqMan Q-RT-PCR.

Gene	Forward Primer 5' – 3'	Reverse Primer 5' – 3'	Probe (Probe number)
AR	CCAGTCCCA ATTGTGTCAAA	TCCCTGGTAC TGTCCAAACG	GGATGGAG (#58)
Cldn3	TGGGAGCT GGGTTGTACG	CAGGAGCAAC ACAGCAAGG	CTGGGCTG (#26)
Cldn11	TGGAGTGG CCAAGTACAGG	GACAATGGCG CAGAGAGC	CTGGCTGG (#20)
Rhox5	AAATGAGCC AGTTGCTGAGG	ATCTGCCTAC CCCCAGGAT	CAGGAGAA (#2)

3.2.5 Western blots

Western blots were performed on proteins extracted from SK11 cells and adult mouse tissues (testis and prostate) using 1x RIPA (section 2.4.1). SK11 cell samples were from untreated cells cultured at 34°C and 39°C, and those incubated with media alone DMSO (vehicle) or DHT (sections 2.2.1 and 2.2.3). Western blots were performed as described in section 2.4.2, protein bands were visualised by fluorescent staining using the LiCor

system. SK11 cell extracts were incubated with antibodies directed against AR, Rhox5, and Sdmg-1 plus β-Actin as a loading control. The primary antibodies used are listed in Table 3-4. The fluorescent secondary antibodies are those listed in

Table 2-5.

Table 3-4: Primary antibodies used on Western blots.

Antigen	Dilution	Species in which antibody was raised	Source
AR	1:200	rabbit	US Biological
Rhox5	1:500	rabbit	Abcam
Sdmg-1	1:1000	rabbit	Gift from Ian Adams (MRC HGU, Edinburgh)
β-Actin (Loading control)	1:5000	mouse	Sigma

3.2.6 Immunohistochemistry

Immunohistochemistry was performed on sections from wild-type mouse testes prepared as described in section 2.5. The expression of AR, Sdmg-1, and SGP-2 was determined in testes from adult and post-natal day 10 mice, and visualised by DAB staining using the methods, reagents and biotinylated secondary antibodies described in Table 2-7 and Table 3-5.

Table 3-5: Primary antibodies used for immunohistochemistry.

Antigen	Dilution	Species in which antibody was raised	Source
AR	1:50	rabbit	Santa Cruz
Sdmg-1	1:1000	rabbit	Gift from Ian Adams (MRC HGU, Edinburgh)
SGP-2	1:250	rabbit	Santa Cruz

3.3 Results

3.3.1 Immunolocalisation of Sertoli cell proteins

Immunoexpression of AR, SGP-2 and Sdmg-1 in adult and day 10 mouse testes was assessed using fixed tissue sections (Figure 3-2, Figure 3-3, and Figure 3-4 respectively). Analysis of day 10 testes was important because SK11 cells were originally derived from mouse testes of this age, and it was essential to demonstrate expression in the equivalent cells *in vivo* before investigating *in vitro* expression.

The testes of day 10 mice were dramatically different from those of adults. The tubules of day 10 mice were much smaller and many had not developed a lumen. The immature tubules had a homogeneous structure with little variation in structure or cell complement between tubules. In adult testes spermatogenesis was apparent with GC at each step of development present, therefore tubules at different stages of the spermatogenesis cycle were identified. The tubules of the adult testes all possessed a lumen, and its size varied with tubule's stage in spermatogenesis.

Expression of AR (Figure 3-2) was detected in testes at both ages within the tubules and in the interstitium, staining was present in the nuclei of Leydig cells (LC), peritubular myoid cells (PTM) and SC, but GC in the adult sections were not stained. In adult testes (panels B and D) the SC nuclei, which stained for AR, were found at the basement of the tubules in contrast to SC of day 10 mice (A and C) where the nuclei were localised towards the centre of the tubules within the epithelium. In the day 10 testes staining for AR was present in SC nuclei in all tubules, AR immunostaining in adult testes was dependent upon the stage of spermatogenesis of the tubules. Tubules at stages VII and VIII in adult mouse testes contained SC with the most intense immunopositive staining for AR in their nuclei.

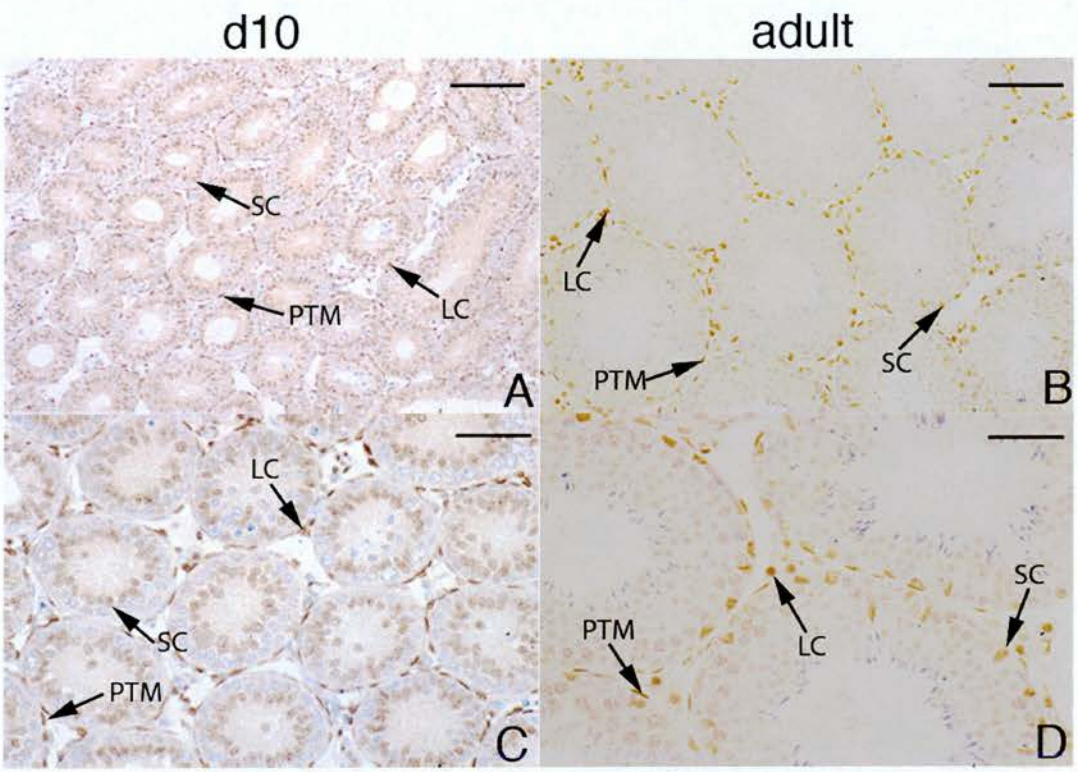


Figure 3-2: Immunostaining of AR in day 10 (A and C) and adult (B and D) mouse testes. Panels A and B are 20x magnification, and panels C and D are 40x magnification. The scale bars represents 100 μm (A and B) and 50 μm (C and D). Arrows indicate positive immunostaining in peritubular myoid cells (PTM), Leydig cells (LC), and Sertoli cell nuclei (SC).

SGP-2 (Figure 3-3) was detected at both ages but was restricted to the SC, and again the distribution differed between ages. Staining in day 10 testes (panel A and B) was concentrated in SC cytoplasm near the centre of the tubule, and lacked the extended cytoplasmic distribution seen in adult testes. In the adult testes (panels C and D) the cytoplasmic location resulted in narrow bands of staining from the base of the tubule in towards its centre, and there was limited stage-dependent variation in intensity of expression.

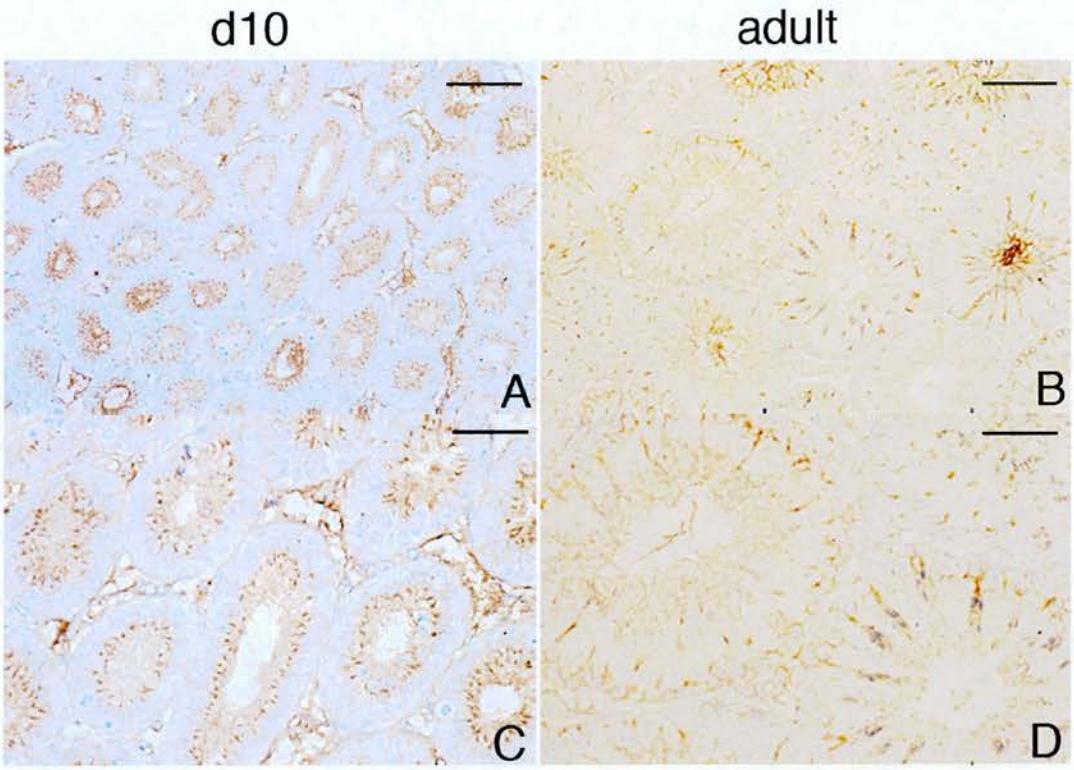


Figure 3-3: Immunostaining of SGP-2 in day 10 (A and C) and adult (B and D) mouse testes. Panels A and B are 20x magnification, and panels C and D are 40x magnification. The scale bars represents 100 µm (A and B) and 50 µm (C and D).

Sdmg-1 staining (Figure 3-4) was present in the tubules of both day 10 and adult testes. In day 10 testes SC cytoplasm was stained strongly from the base to the centre of the tubules surrounding the GC and SC nuclei present. In adult testes Sdmg-1 was also immunolocalised to SC cytoplasm, the staining was in narrow bands from the basement of the tubule inwards towards the lumen in a similar pattern to that of SGP-2.

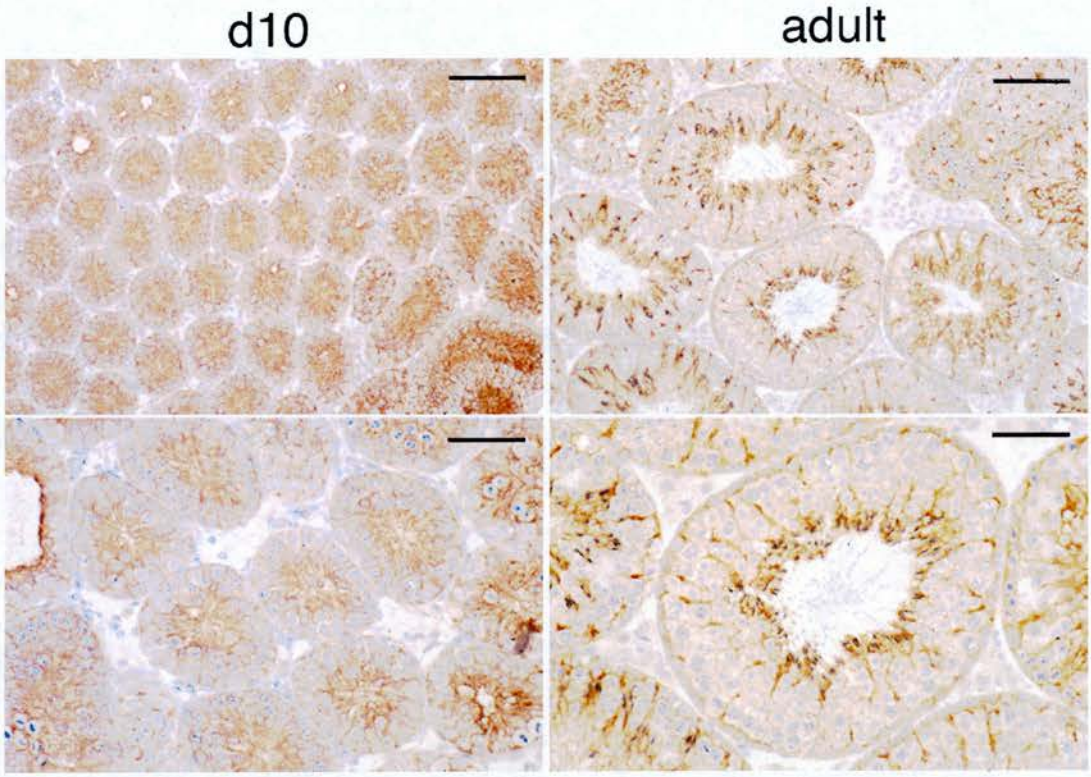


Figure 3-4: Immunostaining of Sdmg-1 in day 10 (A and C) and adult (B and D) mouse testes. Panels A and B are 20x magnification, and panels C and D are 40x magnification. The scale bars represents 100 μm and 50 μm respectively.

3.3.2 Gene expression in SK11 cells at permissive and non-permissive temperatures

3.3.2.1 *SGP-1* and *-2* mRNA

3.3.2.1.1 RT-PCR

SGP-1 (484 bp) and *SGP-2* (460 bp) PCR products were detected in SK11 cells incubated at 34°C, or 39°C for 48 hours, and compared to expression in day 8 and adult mouse testes, plus adult mouse epididymis. Expression of *SGP-1* mRNA (Figure 3-5) appeared higher in cells after incubation at 39°C (lane 3) compared with those at 34°C

(lane 2). *SGP-1* was also expressed in the adult testis and epididymis, and day 8 testis (lanes 1, 4, and 5 respectively), consistent with SC specific expression the amount of mRNA compared to the total testicular RNA was higher at day 8 than in adulthood. In SK11 cells cultured at the 34°C (lane 2, Figure 3-5) expression of *SGP-2* was not detected however mRNA was induced in cells cultured at 39°C (lane 3) and was present in day 8 and adult testes, and epididymis. Expression of *SGP-2* was detected in adult and day 8 testes (lanes 1 and 5), and to a greater extent in adult mouse epididymis (lane 4).

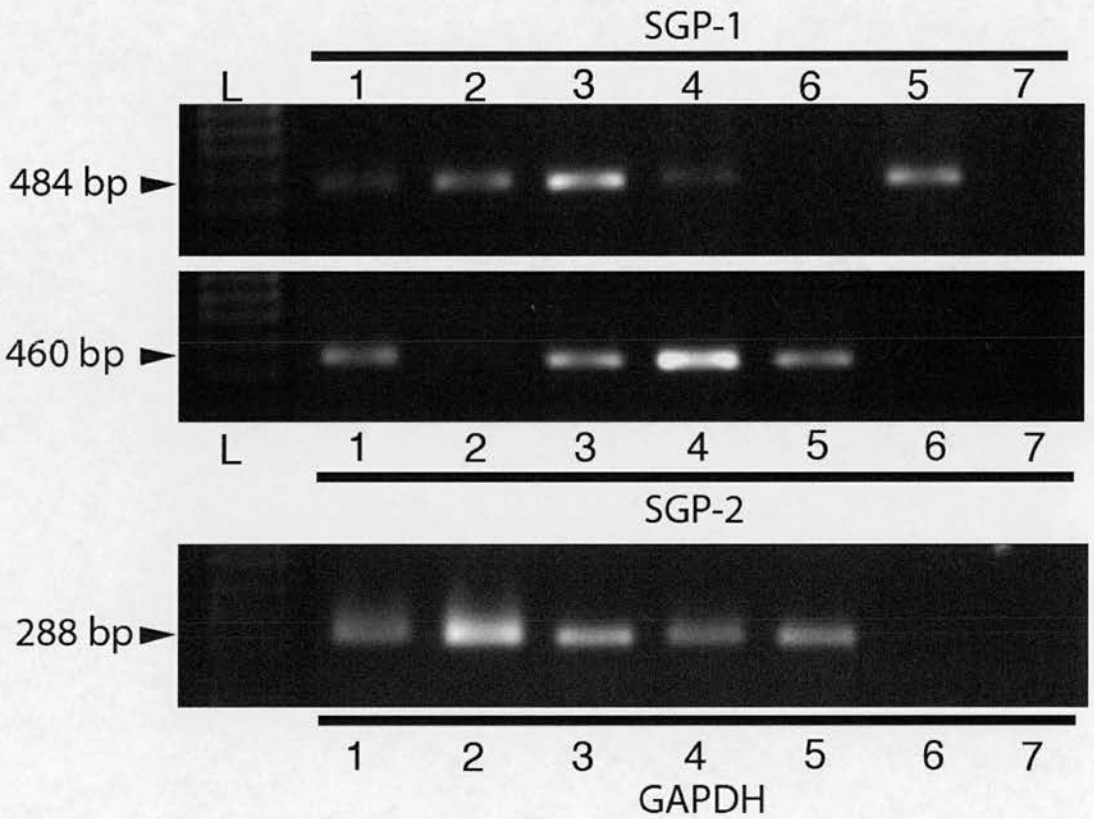


Figure 3-5: Detection of *SGP-1* (484 bp) and *SGP-2* (460 bp) mRNAs. Lane 1 is adult mouse testis (positive control), lane 2 is SK11 cells cultured at 34°C, lane 3 is SK11 cells cultured at 39°C, lane 4 is epididymis, and lane 5 shows immature day 8 mouse testis. Lanes 6 and 7 are negative controls, lane 6 is a reverse transcriptase negative sample, and lane 7 is a water control. L is Hyperladder IV DNA marker and band sizes are marked on the left side of the figure. Expression of GAPDH (288 bp) mRNA in the same samples is include as a control for comparison with SGP-1 and -2 mRNA expression.

3.3.2.1.2 Q-RT-PCR for SGP-2 in SK11 cells incubated with testosterone

The mRNA expression of *SGP-2* in SK11 cells cultured at 34°C, or for 48 hours at 39°C, was also measured by TaqMan Q-RT-PCR (Figure 3-6) to quantify the difference in expression detected by RT-PCR (shown in Figure 3-5). The impact of testosterone ($1x10^{-9}$ - $1x10^{-6}$ M) was also quantified. Expression of *SGP-2* mRNA was increased by 3 - 4.5-fold when cells were cultured at 39°C, compared to untreated cells cultured at 34°C. Treatment of cells, cultured at 34°C or 39°C, with testosterone did not induce a statistically significant change in *SGP-2* mRNA expression at any concentration. Statistical analysis by 2-way ANOVA confirmed these observations and reported that culture temperature had a highly significant effect on *SGP-2* expression ($p<0.0001$), but that testosterone treatment had no significant effect ($p=0.55$). A Post-hoc test showed that the difference in *SGP-2* mRNA expression between cells cultured at 34°C and 39°C was only ($p<0.01$) highly significant in cells cultured with 1 x 10^{-6} M testosterone (indicated by ** in Figure 3-6).

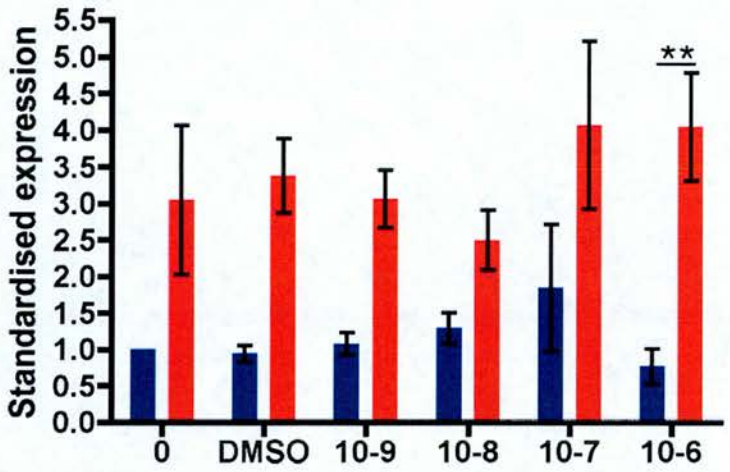


Figure 3-6: TaqMan for *SGP-2* mRNA in SK11 cells grown at 34°C (blue) and 39°C (red) and treated with testosterone ($1x10^{-9}$ – $1x10^{-6}$ M) for 48 hours. The control (0) cells were incubated in phenol-red free media alone, and DMSO is the vehicle control. Expression is presented relative to that of control SK11 cells cultured at 34°C. Bars represent average expression ± SEM, for n=3 experiments. 2-way ANOVA reported that culture temperature had a significant effect ($p<0.0001$) on SGP-2 expression. Treatment with media, DMSO or testosterone did not affect expression significantly ($p=0.55$). ** = $p<0.01$.

3.3.2.1.3 Western

SGP-2 protein was detected in samples extracted from SK11 cells incubated at 34°C and 39°C (Figure 3-7). Panel A shows the fluorescently labelled proteins bands of SGP-2 (70 kDa) and β-actin (42 kDa), extracts from cells incubated at 34°C (lanes 1) were associated with less total SGP-2 than extracts from cells at 39°C (lanes 2). Panel B shows quantification of SGP-2 expression in cell extracts, standardised to β-actin; approximately 80% more SGP-2 was detected in cells incubated at 39°C, than at 34°C. Statistical analysis of these data was performed using A Mann Whitney analysis, and revealed no significant difference ($p=1.00$) in expression between culture temperatures.

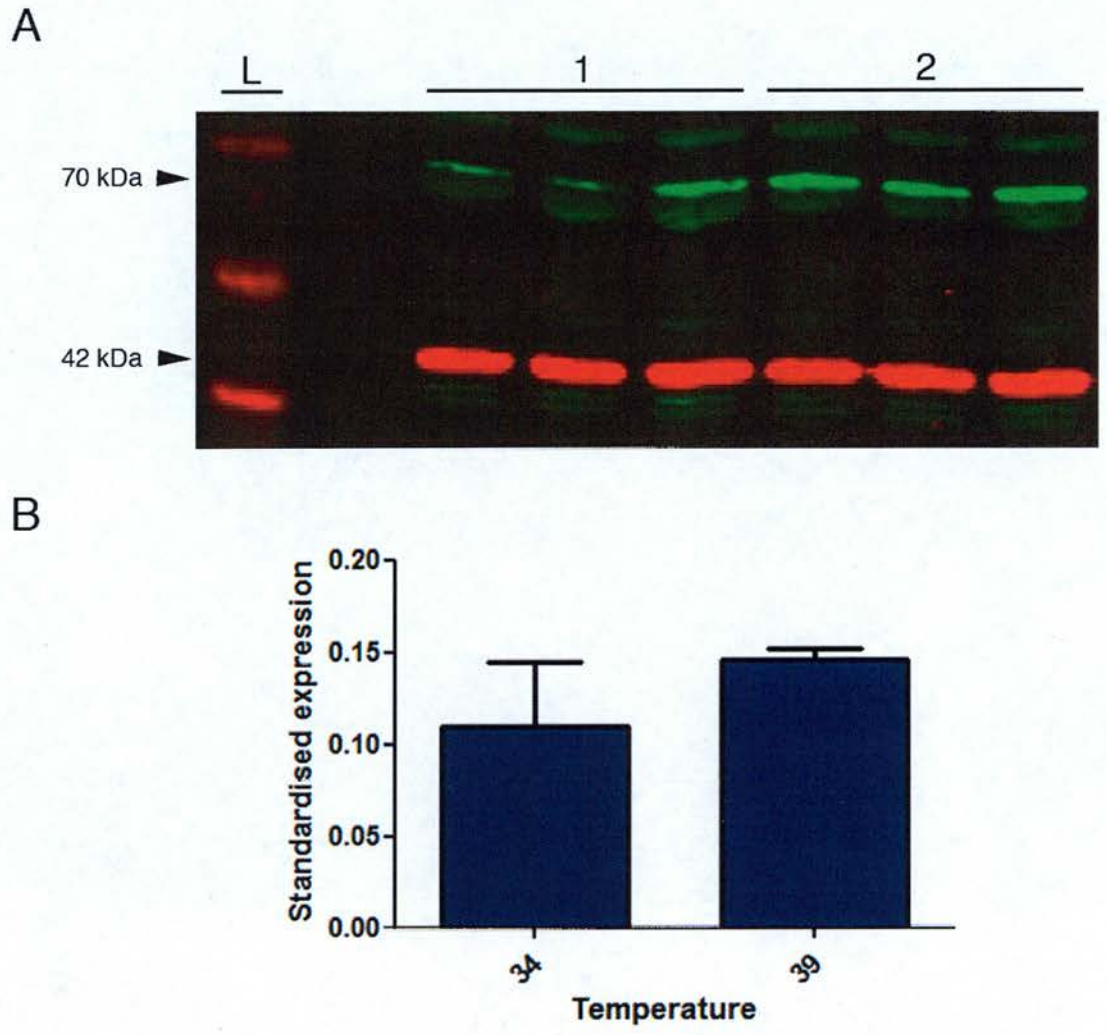


Figure 3-7: Expression of SGP-2 protein in SK11 cells cultured at 34°C or 39°C. Panel A shows fluorescently labelled bands associated with SGP-2 (70 kDa, green) and β-actin (42 kDa, red). Proteins were extracted from SK11 cells incubated at 34°C (lanes 1) or 39°C (lanes 2), lane L contained SeeBlue protein ladder. Panel B is quantification of SGP-2, standardised to β-actin, detected in n=3 sets of cell extracts. No significant difference (p=1.00) in expression between cells cultured at 34°C and 39°C was detected by the Mann Whitney test.

3.3.2.2 Sdmg-1

3.3.2.2.1 Western

Sdmg-1 protein was detected in protein extracts from SK11 cells incubated at 34°C (lanes 1) or 39°C (lanes 2), the fluorescently labelled bands associated with Sdmg-1 (80 kDa, green) and β-actin (42 kDa, red) are shown in panel A (Figure 3-8). The size of Sdmg-1 protein was predicted from its nucleotide sequence to be 49 kDa (Best et al., 2008), however shift in the gel is reduced by N-glycosylation. Sdmg-1 shift can be reduced to equivalent to that of a 110-120 kDa protein, shift of the protein has not been reduced this dramatically in the SK11 cell samples but fluorescence of the putative Sdmg-1 bands is strong indicating specific-binding. The Sdmg-1 expression appeared similar at both temperatures, to be certain Sdmg-1 was quantified and standardised to β-actin in each extract. Panel B shows standardised Sdmg-1 protein expression, approximately 30% more Sdmg-1 was detected in extracts from cells incubated at 39°C, compared to 34°C. A Mann Whitney statistical analysis revealed no significant difference ($p=0.40$) in expression between cells cultured at each temperature.

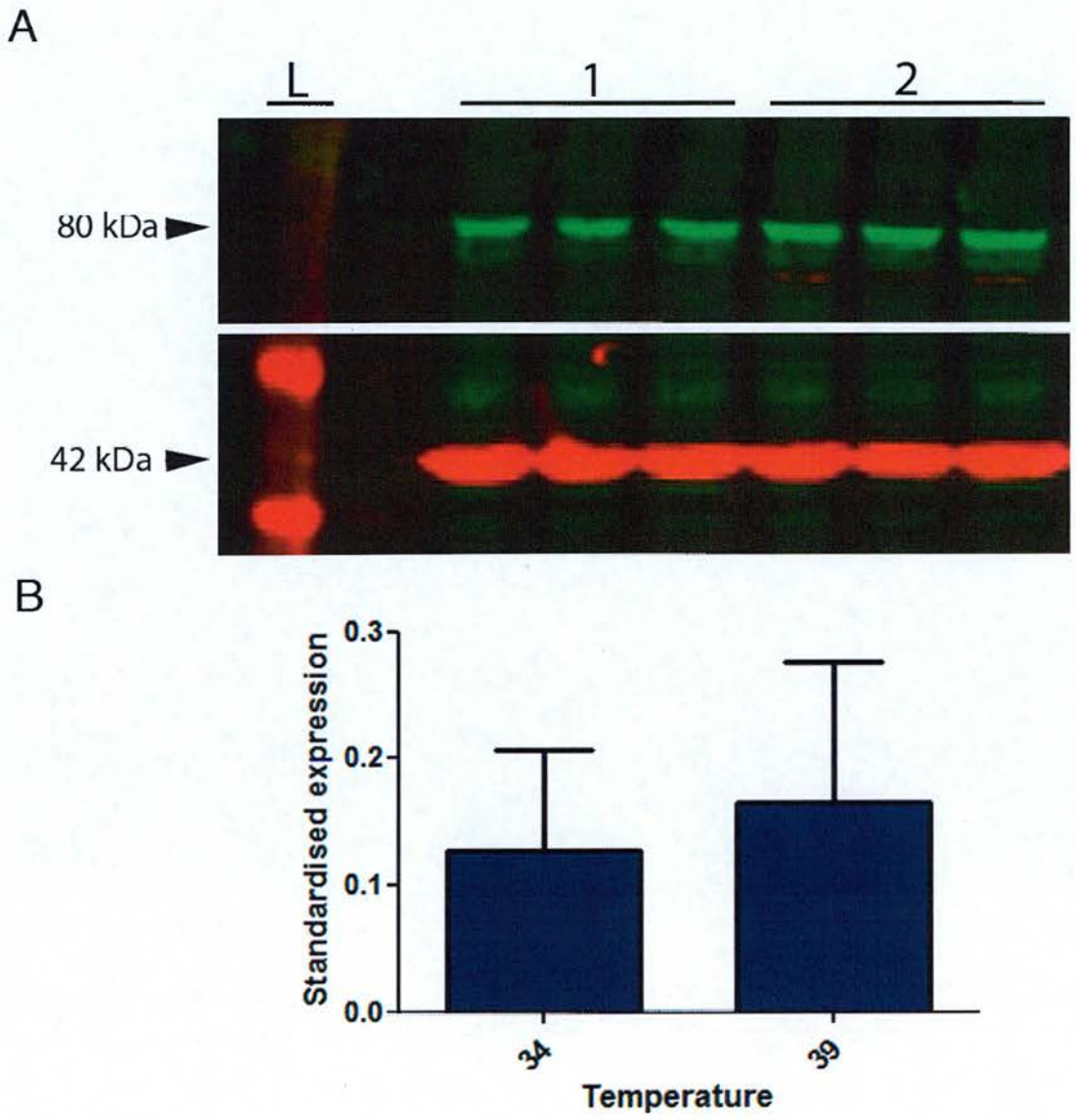


Figure 3-8: Sdmg-1 protein in extracts from SK11 cells incubated at 34°C (lanes 1) or 39°C (lanes 2). Sdmg-1 was detected using fluorescent western blotting, panel A shows the fluorescent bands associated with Sdmg-1 (80 kDa, green) and β-actin (42 kDa, red), and in lane L the SeeBlue protein ladder. Panel B shows quantification of Sdmg-1 standardised to β-actin in 3 sets of cell extracts, a Mann Whitney detected no significant difference (p=0.40) between cells cultured at 34°C and 39°C.

3.3.2.3 Androgen receptor

3.3.2.3.1 RT-PCR for *AR* in SK11 cells incubated with DHT

Weak expression of *AR* mRNA (171 bp) was detected by RT-PCR in SK11 cells cultured at 34°C (top panel) or 39°C (bottom panel), and the level of expression appeared similar at both temperatures (Figure 3-9). Incubation of cells with DMSO (vehicle, lanes marked 3) or $1x10^{-7}$M DHT (lanes marked 2) for 48 hours did not alter expression of *AR* compared to cells cultured in media alone (lanes marked 1).

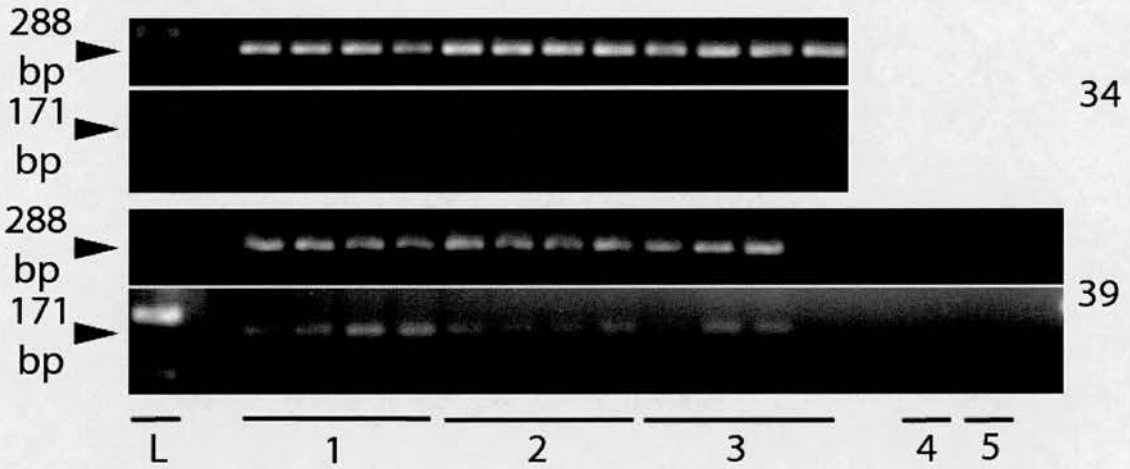


Figure 3-9: Detection of *AR* mRNAs in SK11 cells incubation at 34°C or 39°C with media, DHT or DMSO for 48 hours. *AR* (171 bp) expression was detected in control SK11 cells (1, cultured in media) and those incubated with, 10^{-7} M DHT (2) and DMSO (3). Lane 4 shows a RT-negative control, and lane 5 is a water control. L is Hyperladder IV DNA marker and band sizes are marked on the left side of the figure. Expression of GAPDH mRNA (288 bp) in the same samples is included as a control.

3.3.2.3.2 Q-RT-PCR for Ar in SK11 cells incubated with testosterone

Expression of *AR* mRNA in SK11 cells was quantified by TaqMan Q-RT-PCR to assess whether culture temperature or incubation with androgens had an impact on *AR* expression, which had not been detected by semi-quantitative RT-PCR. The quantification from the TaqMan is shown in Figure 3-10, SK11 cells cultured at 34°C (blue bars) and the differentiated cells at 39°C (red bars). However overall *AR* mRNA

expression was increased by culture at 34°C compared to 39°C (p=0.01). In addition, Bonferoni post-hoc test found no statistically significant difference in *AR* expression between cells cultured at 34°C or 39°C undergoing the same treatment. Incubation with media, DMSO or testosterone did not affect *AR* expression significantly (p=0.56).

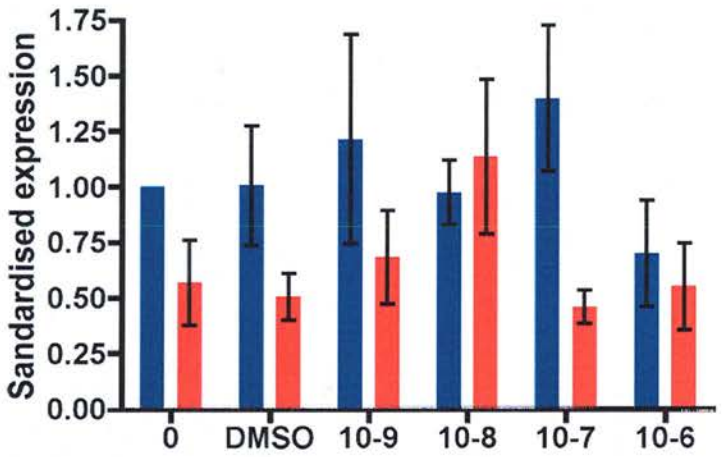


Figure 3-10: TaqMan quantification of *AR* mRNA in SK11 cells incubated for 48 hours with testosterone (10^{-9} to 10^{-6} M), media (control, 0), or DMSO. Cells were cultured at 34°C (blue) or 39°C (red). *AR* expression was standardised to SK11 cells incubated in media at 34°C. Bars represent average expression ± SEM, for n=3 experiments. Expression of *AR* was significantly affected by culture temperature (p=0.01), but incubation with media, DMSO or testosterone did not have a significant effect (p=0.56), when analysed by 2-way ANOVA.

3.3.2.3.3 Western

Androgen receptor protein was detected in SK11 cells incubated at 34°C or 39°C (Figure 3-11). Fluorescently labelled AR (110 kDa, green) and β-actin (42 kDa, red) protein bands detected using a Western blot are shown in panel A, a modest reduction in total AR was seen in cells incubated at 39°C. The amount of AR detected in extracts from three experimental runs was quantified and standardised to β-actin. The average standardised AR detected in cells at each temperature is presented, statistical analysis by

Mann Whitney test detected no significant difference (p=0.20) in AR protein expression between cells cultured at 34°C and 39°C.

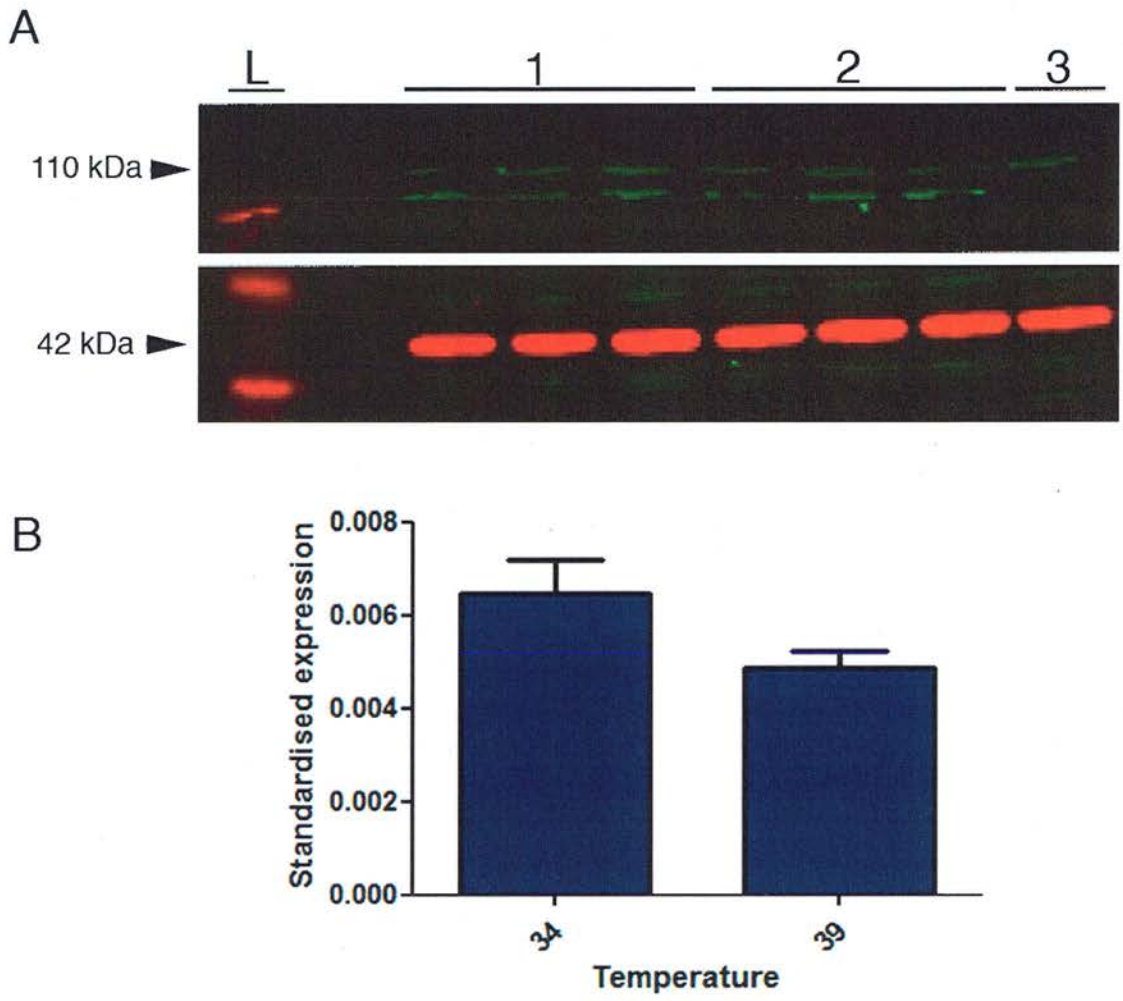


Figure 3-11: Androgen receptor protein expression in SK11 cells cultured at 34°C (lanes 1) or 39°C (Lanes 2). Panel A shows fluorescently labelled AR (110 kDa, top green band only) and β-actin (42 kDa, red) protein bands detected by western blotting in the cell extracts. Lane 3 contained protein extracted from adult mouse testis and lane L was SeeBlue protein ladder. Panel B shows quantification of AR protein detected in cell incubated at 34°C and 39°C. AR level was standardised to β-actin, and is the average of 3 runs. Statistical analysis by Mann Whitney test reported no significant difference (p=0.20).

3.3.2.4 Rhox5

3.3.2.4.1 RT-PCR for *Rhox5* in SK11 cells incubated with DHT

Expression of *Rhox5* was detected by RT-PCR in adult mouse testis (positive control) and samples extracted from cells cultured at 34°C or 39°C in the absence or presence of DHT (Figure 3-12). Although mRNA was readily detected in all samples it was not possible to determine whether culture temperature or incubation with DHT had a significant impact on expression of *Rhox5* mRNA.

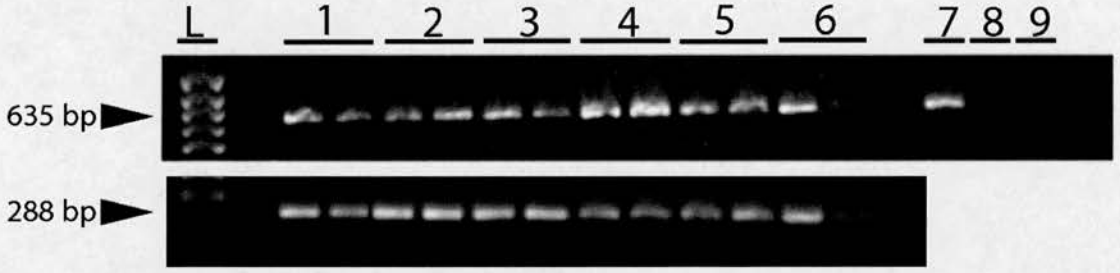


Figure 3-12: *Rhox5* (635 bp) mRNA detected using RT-PCR in SK11 cells cultured at 34°C (lanes 1-3) and 39°C (lanes 4-6). Cells were incubated with media (lanes 1 and 4), 10^{-7} M DHT (lanes 2 and 5), or DMSO (lanes 2 and 6) for 48 hours. Lane 7 is a positive control (adult mouse testis), lane 8 is a reverse transcriptase negative adult mouse testis sample, and lane 9 is a water control. L is Hyperladder IV DNA marker and the band size is marked on the left side of the figure. Expression of GAPDH mRNA (288 bp) in the same samples is included as a control.

3.3.2.4.2 Q-RT-PCR for Rhox5 in SK11 cells incubated with testosterone

The expression of *Rhox5* by SK11 cells was also quantified by TaqMan RT-PCR (Figure 3-13). Culturing SK11 cells at 39°C resulted in a significantly greater expression of *Rhox5* mRNA than in cells cultured at 34°C ($p<0.0001$), expression in SK11 cells cultured at 39°C ranged from 2-3 fold greater than in cells cultured at 34°C. Changes in *Rhox5* mRNA expression between cells in different culture conditions were analysed by Bonferoni post-hoc test. When cells were cultured in media, or in the presence of

testosterone at $1x10^{-7}$ M, $1x10^{-6}$ M or $1x10^{-9}$ M there was a significant increase in *Rhox5* mRNA expression when cultured at 39°C, compared to 34°C. Treatment with DMSO (vehicle control) or doses of testosterone between $1x10^{-9}$ M and $1x10^{-6}$ M did not change *Rhox5* expression significantly from that in cells cultured in media (p=0.91).

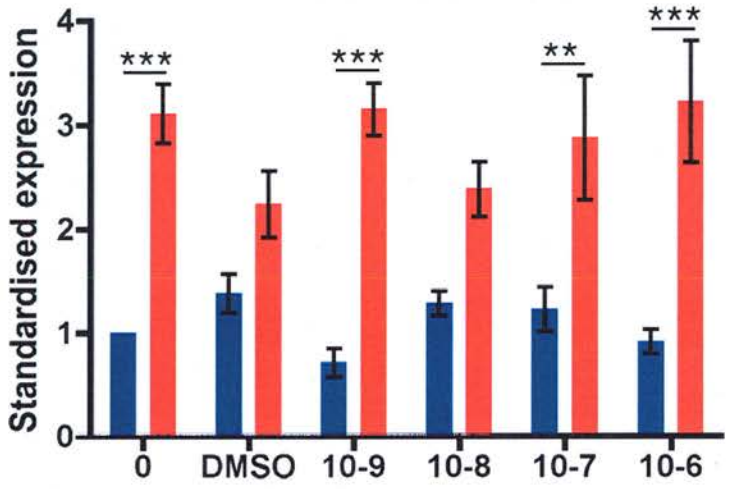


Figure 3-13: Quantification of *Rhox5* mRNA by SK11 cells by Taqman Q-RT-PCR. SK11 cells were incubated with testosterone (10^{-9} - 10^{-6} M), media (control), or DMSO (vehicle control) for 48 hours at 34°C (blue) and 39°C (red). Expression was standardised to that of SK11 cells incubated with media at 34°C. Bars represent average expression ± SEM, for n=3 experiments. A 2-way ANOVA demonstrates a significant difference (p<0.0001) in expression between culture temperatures, but no difference due to testosterone treatment (p=0.91). ** = p<0.01 and *** = p<0.001.

3.3.2.4.3 Western

Expression of Rhox5 protein was detected in SK11 cells by fluorescent Western blotting (top panel, Figure 3-14). Included on the Western blots is the positive control tissue, adult mouse testis (lane 7). Expression of Rhox5 (30 kDa, bottom green band on Western blot) and β-actin (42 kDa, top green band) were quantified, and Rhox5 expression was normalised to the β-actin in each sample. Expression in each sample was expressed relative to SK11 cells incubated with media at 34°C, and the average expressions in cells cultured at 34°C (blue) or 39°C (red) and mouse tissues (purple bars)

are shown below in Figure 3-14 (bottom panel). Expression of Rhox5 protein was significantly increased by culture at 39°C ($p<0.002$), although expression only reached a maximum of around 1.6-fold the level detected in SK11 cells incubated with media at 34°C. Bonferoni post-hoc test detected a significant difference in expression specifically between cells cultured at 34°C and 39°C incubated in media alone ($p<0.05$). Treatment with DHT or DMSO had no significant impact on expression of Rhox5 protein ($p=0.61$).

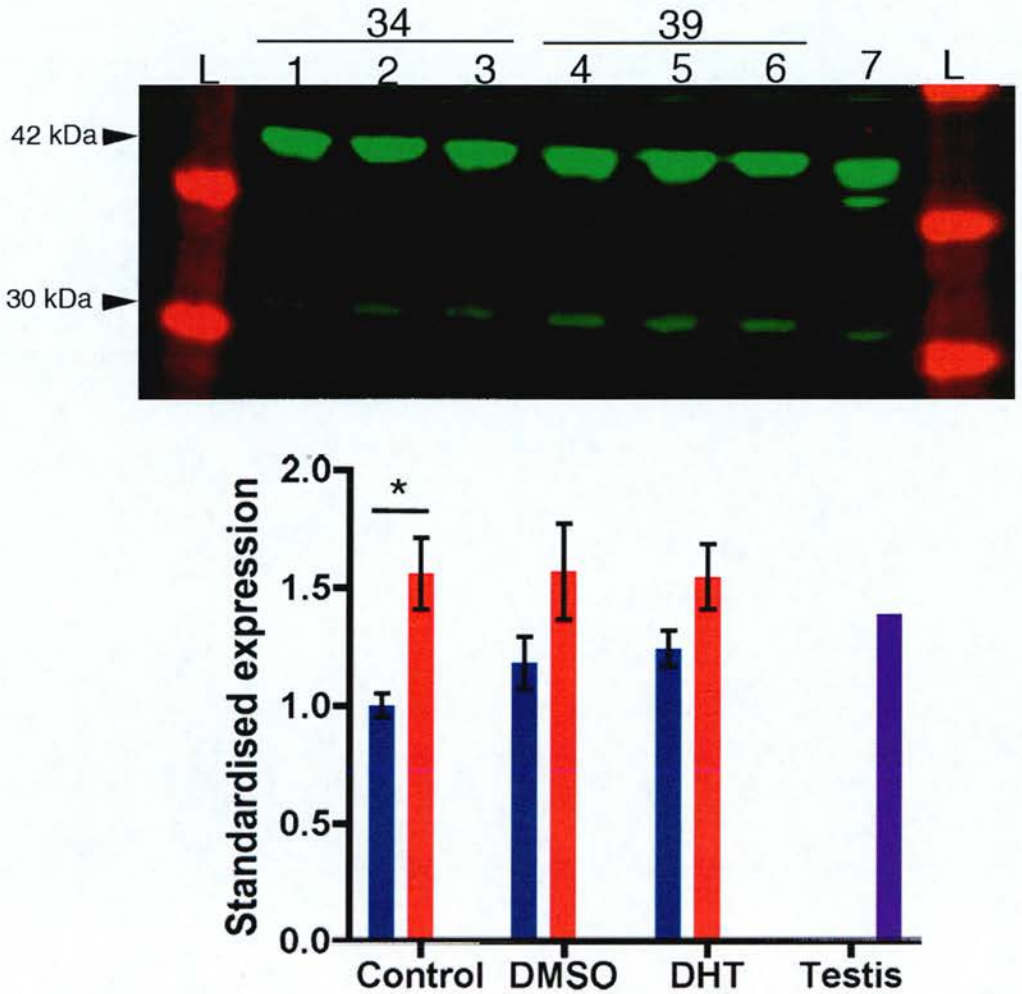


Figure 3-14: Western blot analysis of Rhox5 protein detected in SK11 cells cultured at 34°C or 39°C, and treated with DHT. The top panel shows fluorescently labelled β-actin (42 kDa, top green bands) and Rhox5 (23 kDa, bottom green bands) following Western blotting. Cells were incubated with media (lanes 1 and 4), DMSO (lanes 2 and 5), or 10^{-7}M DHT (lanes 3 and 6) for 48 hours. Lane 7 is protein from adult mouse testis, and L is SeeBlue protein marker and protein band sizes (30 kDa). The lower panel shows quantified Rhox5 expression (standardised to β-actin) in cells cultured at 34°C (blue) or 39°C (red). Rhox5 expression was plotted relative to expression in SK11 cells incubated in media at 34°C. The purple bars show expression of Rhox5 in testis (positive control tissue). Bars represent mean expression ± SEM, for n=4 samples (Control and DHT), n=2 sample (DMSO), or n=1 (testis). Analysis by 2-way ANOVA demonstrated a significant difference between culture at 34°C and 39°C ($p<0.002$), but no significant difference between control, DMSO or DHT treatments ($p=0.61$). * = $p<0.05$.

3.3.3 Gene expression in SK11 cells following transfection with AR cDNA

As levels of *AR* mRNA in SK11 cells appeared to be low and there was no detectable androgen response, cells were transfected with between 300ng and 1.5µg of mouse *AR* (*mAR*) cDNA. It was hoped that expression of the transfected *mAR* plasmid would result in a detectable androgen-dependent response.

3.3.3.1 Androgen receptor

3.3.3.1.1 Q-RT-PCR

Expression of *mAR* was quantified by TaqMan in SK11 cells transiently transfected with a *mAR* cDNA and cultured ± $1x10^{-7}$M DHT at 34°C for 48 hours (Figure 3-15). Transfection successfully increased the amount of *AR* mRNA detected. In cells transfected with 500 ng *mAR* cDNA was significantly greater than in untransfected cells ($p<0.01$), and this significance rose when ≥750 ng were introduced ($p<0.0001$). The fold increases in expression detected following transfection were in the tens to hundreds of thousands compared to untransfected cells. Treatment of the cells with DHT did not affect expression of *AR* significantly in transfected or untransfected cells, even when large quantities of *AR* cDNA were introduced ($p=0.12$).

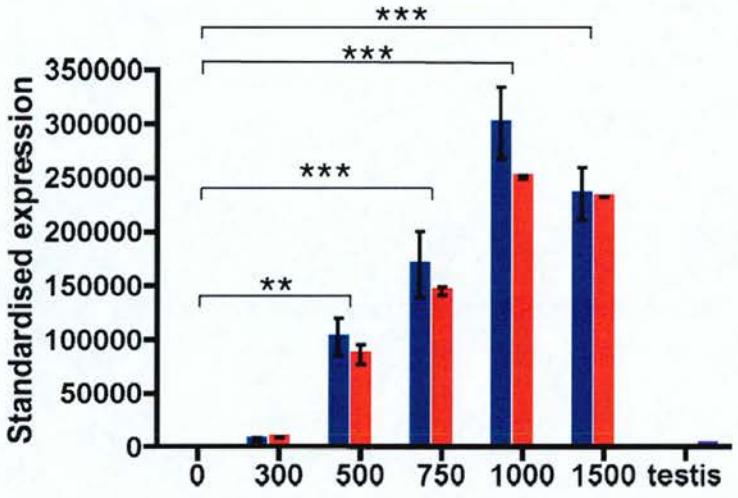


Figure 3-15: *AR* mRNA quantification in SK11 cells transfected with 0 – 1500 ng *mAR* cDNA per well. Cells were cultured for 48 hours with DMSO (blue) or 10^{-7} M DHT (red) at 34°C. Expression was assessed by Taqman Q-RT-PCR, and expression levels were standardised to *mAR* expression in untransfected SK11 cells incubated with DMSO (valued at 1). Adult mouse testis cDNA (purple) was included for comparison. The graph shows mean ± SEM, based on n=2 samples. Statistical analysis by 2-way ANOVA demonstrated that expression of *AR* was affected significantly ($p<0.0001$) by the quantity of *mAR* plasmid transfected into the cells, however expression level was not affected by DHT treatment ($P=0.12$). ** = $p<0.01$ and *** = $p<0.001$.

3.3.3.1.2 Western

The expression of AR protein (110 kDa) detected in SK11 cells transfected with mAR cDNA (300 – 1500 ng/well) was quantified by fluorescent Western blotting in cells incubated with DHT ($1x10^{-7}$ M) or media alone for 48hours at 34°C (Figure 3-16). AR protein expression was normalised to β-actin in each well, and in panel B is presented relative to expression in untransfected cells incubated in media. AR protein was not detected in untransfected cells, but all transfected cells incubated with DHT expressed detectable amounts of AR. Cells transfected with 300 ng *mAR* and incubated with DHT did not give a visible fluorescence band in panel A, but were sufficiently labelled for

detection with the Odyssey scanner and quantification for panel B. AR protein detected in cells not incubated with DHT ranged from equal to that found in untransfected cells to ~25-fold more. In cells incubated with DHT fold increases ranged from ~5-250 fold compared to untransfected cells incubated with media. Expression of AR protein was only detected in the absence of DHT in cells transfected with ≥500 ng/well cDNA, and AR protein expression was significantly greater than in untransfected cells when ≥500ng cDNA were introduced ($p<0.0001$). The amount of AR protein detected was significantly greater in cells incubated in DHT than those incubated in media ($p<0.0001$), specifically in cells transfected with 500 ng/well ($p<0.01$) and 750-1500 ng/well ($p=0.0001$). There was a positive association between the quantity of cDNA introduced into the cells and the amount of AR protein detected ($p<0.0001$), but the increase was more dramatic in DHT-treated cells (significant interaction between cDNA quantity and DHT treatment, $p<0.0001$). The amount of AR protein detected in SK11 cells transfected with 500 or 750 ng/well cDNA and incubated with DHT was similar to expression detected in adult mouse prostate.

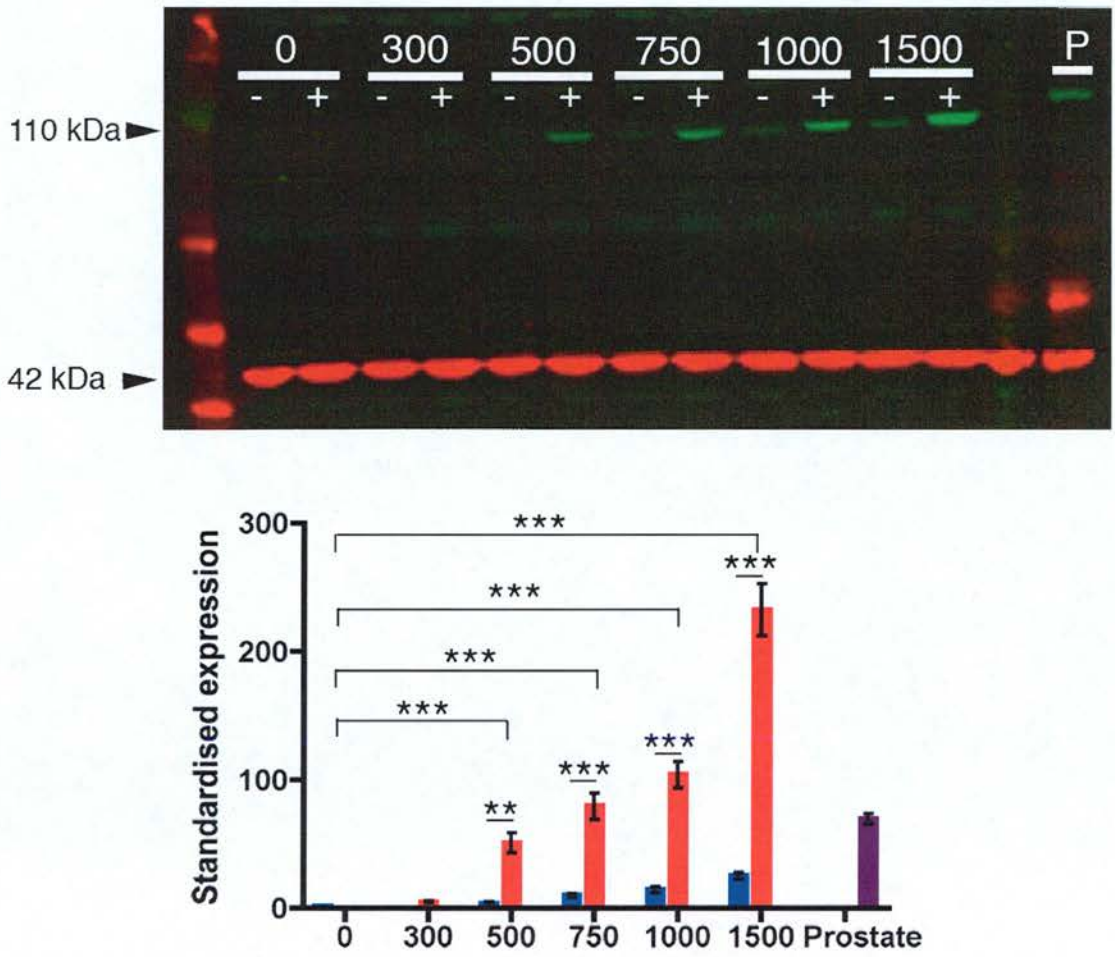


Figure 3-16: Detection of AR protein (110 kDa) in SK11 cells transfected with 0 - 1500ng *mAR* cDNA per well. Cells were incubated with 10^{-7} M DHT or media alone at 34°C. Top panel shows mAR (green) and β-actin (42 kDa loading control, red) expression following transfection of each quantity of plasmid into cells incubated with (+) or without (-) 10^{-7}M DHT. On the left of the image is See-blue protein ladder and protein band sizes (kDa) and on the far right is prostate (P, positive control tissue). The bottom panel shows standardised mAR expression (mAR/β-actin) in cells incubated in media alone (blue) or $1x10^{-7}$M DHT (red), and adult mouse prostate (purple) relative to expression in untransfected cells incubated in media. The graph shows mean ± SEM, based on n=3 samples. A 2-way ANOVA reported a significant differences between cells transfected with different quantities of mAR plasmid and between cells treated DHT and media, the analysis also indicated interaction between these factors resulting in a greater than cumulative effect ($p<0.0001$, all three comparisons). ** = $p<0.01$ and *** = $p<0.001$.

3.3.3.2 Rhox5-luc reporter assay

The functionality of AR protein was investigated by co-transfection of 500 ng *mAR* together with the *Rhox5(*ARE*)-luc* and *Renilla* plasmids into SK11 cells cultured at 34°C. If the transfected cells express functional mAR protein then incubation with DHT should induce luciferase expression following binding of AR to the ARE. Transfected SK11 cells were incubated with transfection media plus FBS alone, DMSO or $1x10^{-10}$ – $1x10^{-7}$ M DHT for 48 hours, expression of firefly luciferase was standardised to renilla expression in each sample, and standardised firefly luciferase expression relative to expression in cells cultured in media alone is shown in Figure 3-17. Treatment of cells with DHT had a significant impact on the amount of luciferase ($p=0.01$, Kruskal-Wallis test), raising expression more than 5-fold over cells cultured in media alone. Post-hoc Dunn's multiple comparison test, revealed that there was a significant difference in expression between cells treated with 1×10^{-9} M DHT and the control cells treated with media alone and DMSO ($p<0.05$).

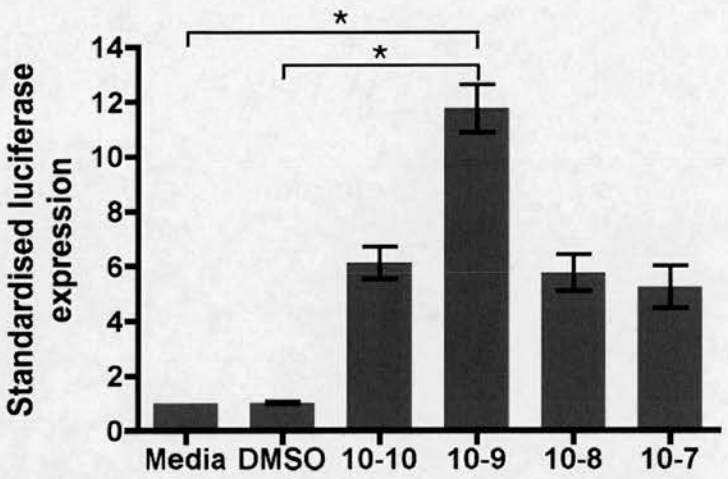


Figure 3-17: Expression of luciferase protein in response to activation of Rhox5-luc, in SK11 cells transfected with mouse *AR* plasmid. SK11 cells transfected with *mAR*, *Rhox5-luc* and *Renilla* were treated with media alone, DMSO or DHT ($1x10^{-10}$ – $1x10^{-7}$ M) at 34°C for 48 hours. Expression is shown relative to luciferase in cells cultured in media. Bars represent mean luciferase expression ± SEM for n=3 experiments. Analysis using the non-parametric Kruskal-Wallis test demonstrated a significant difference between treatments (p=0.01). * = p<0.05.

3.3.3.3 Expression Rhox5 mRNA

Expression of *Rhox5* mRNA was quantified by TaqMan in SK11 cells transfected with 300-1500 ng *mAR* cDNA/well and treated with $1x10^{-7}$M DHT for 48 hours at 34°C (Figure 3-18). Expression of *Rhox5* mRNA in the untransfected and transfected SK11 cells was high in comparison to the adult mouse testis sample. *Rhox5* expression was significantly altered by transfection with *mAR* cDNA ($p<0.01$, 2-way ANOVA), the highest expression was seen in cells transfected with 500 ng/well and the lowest in control cells (untransfected cells incubated with DMSO). *Rhox5* mRNA expression was raised compared to control cells following transfection with 500 ng ($p< 0.01$), or 750 ng and 1000 ng ($p<0.05$), although the greatest fold-changes were <2. *Rhox5* mRNA expression was not altered significantly by incubation with DHT ($p<0.62$), even in cells transfected with 500 ng/well *mAR*, which resulted in significant *Rhox5* promoter activation as described in section 3.3.3.2.

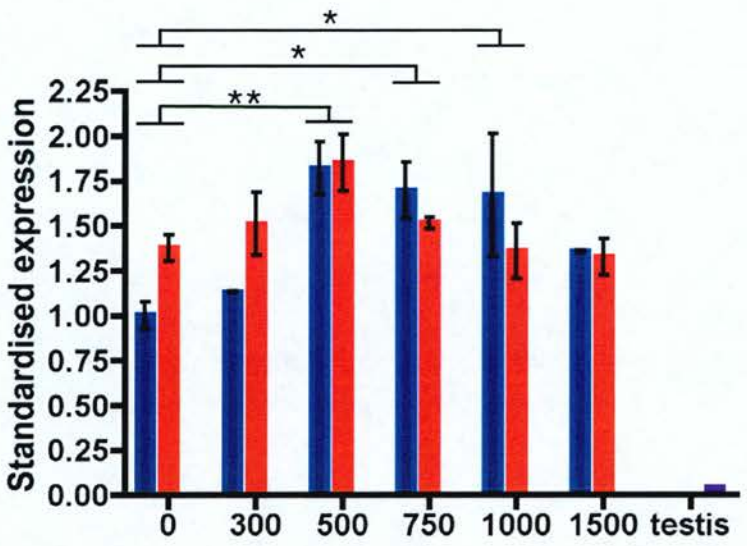


Figure 3-18: Quantification of *Rhox5* mRNA in SK11 cells transfected with between 0 – 1500 ng *mAR* plasmid per well. Cells were cultured for 48 hours with DMSO (blue) or 10^{-7} M DHT (red) at 34°C. Expression was detected by Taqman Q-RT-PCR, and standardised to *Rhox5* expression in untransfected (0) SK11 cells incubated with DMSO. A sample of adult mouse testis cDNA (purple) was included in the analysis. The graph shows standardised mean ± SEM, based on n=2 samples. The quantity of mAR cDNA significantly affected *Rhox5* expression ($p<0.01$), but DHT treatment did not have a significant effect ($p=0.62$). * = $p<0.05$, and ** = $p<0.01$.

In order to determine whether there was a time-dependent change in *Rhox5* mRNA in cells transfected with 500 ng/well *mAR* cDNA cells were incubated for 6 to 48 hours with $1x10^{-9}$ M DHT, DMSO, or transfection media plus FBS. These conditions should ensure substantial AR protein expression and the optimal DHT concentration to stimulate the *Rhox5* proximal promoter and cover a time period in which *Rhox5* mRNA expression can be expected to change. *Rhox5* mRNA expression relative to control cells cultured for 6 hours in media alone is shown in Figure 3-19. Expression rose from 16 hours to 24 hours and declined by 48 hours, all changes were within 2-fold of control. Statistical analysis by 2-way ANOVA reported that the differences in expression between time points were not significant ($p=0.11$), nor did treatment with DHT have a significant effect ($p=0.81$).

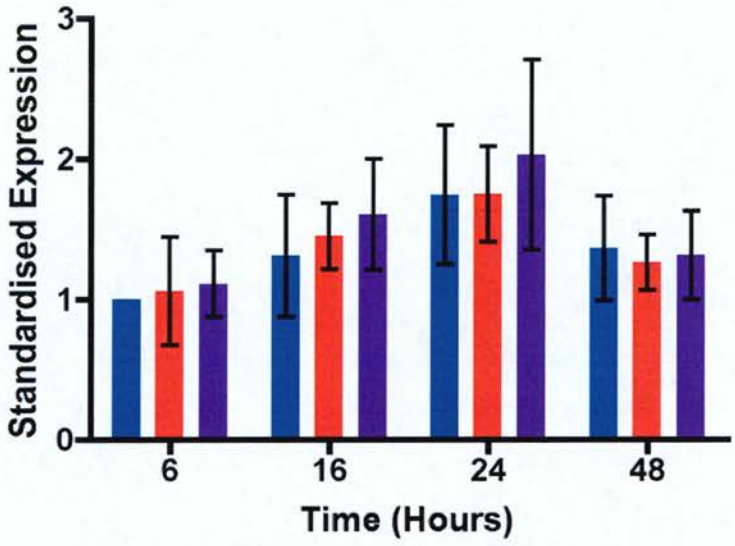


Figure 3-19: Time course of *Rhox5* mRNA detected in SK11 cells transfected with mouse AR. Expression of *Rhox5* mRNA was quantified by Q-RT-PCR in samples from cells incubated at 34°C for between 6 and 48 hours with transfection media plus FBS (blue), DMSO (red), or 1 x10^{-9} M DHT (purple). Expression in each sample was standardised to that of cells incubated for 6 hours with FBS. Bars represent mean expression ± SEM from 3 independent experiments. Statistical analysis by 2-way ANOVA revealed no significant differences between time points ($p = 0.11$) or treatment with media, DMSO or DHT ($p= 0.81$).

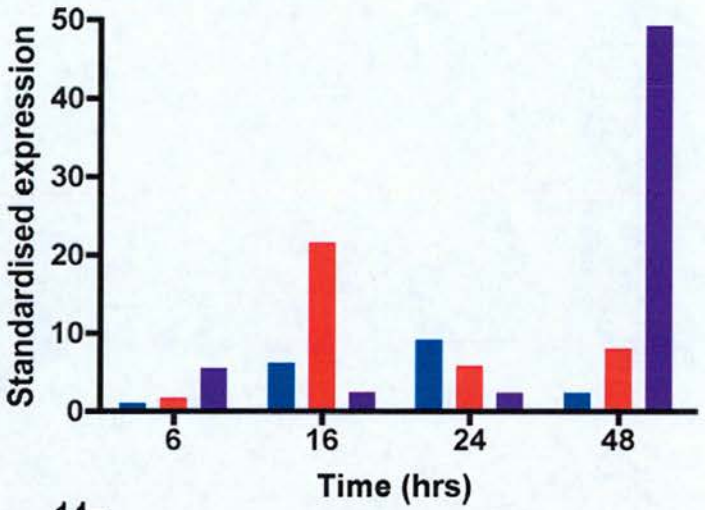
3.3.3.4 Expression of junctional mRNAs

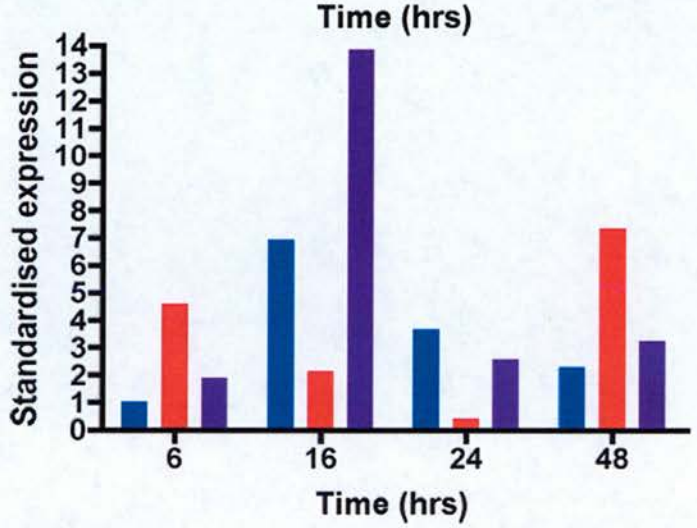
SK11 cells were transfected with 500 ng/well of *mAR* cDNA and incubated with 1x10^{-9}M DHT, DMSO or transfection media plus FBS for 6 – 48 hours at 34°C. *Claudin3* (*Cldn3*) and *claudin11* (*Cldn11*) mRNA expression were quantified by TaqMan Q-RT-PCR.

3.3.3.4.1 *Claudin3* Q-RT-PCR

Expression of *Cldn3* in cells from 3 independent experiments was standardised to expression in cells incubated for 6 hours in media within the same experiment; the results were highly variable and are shown separately in Figure 3-20. The amounts of mRNA detected were highly variable between experiments. Fold changes ranging from

less than 1 to close to 50 were detected and no common pattern of expression was seen between graphs. None of the time points or treatments were consistently associated with changes in expression.


Standardised expression
50
40
30
20
10
0
6
16
24
48
Time (hrs)


Standardised expression
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
6
16
24
48
Time (hrs)

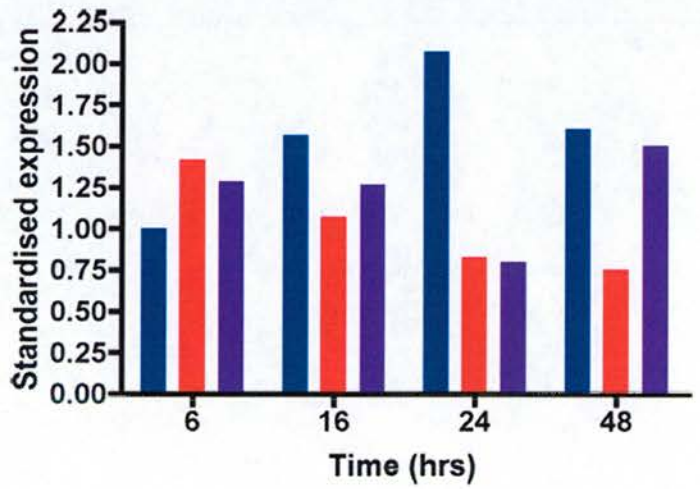

Standardised expression
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
6
16
24
48
Time (hrs)

Figure 3-20 (previous page): Quantification of *claudin3* (*Cldn3*) mRNA in SK11 cells transfected with mouse *AR*. Expression of *Cldn3* in SK11 cells, cultured at 34°C and incubated with transfection media plus FBS (blue), DMSO (red), and 1 x 10^{-9} M DHT (purple), was detected by Q-RT-PCR. Expression was quantified at 6, 16, 24 and 48 hours. Three independent experiments were undertaken and the results of each are shown separately above. Bars show the average expression, from 2 wells per treatment per time point, standardised to expression in SK11 cells incubated with transfection media plus FBS for 6 hours.

3.3.3.4.2 *Claudin11* Q-RT-PCR

SK11 cells were transfected as described above and incubated with DMSO; $1x10^{-9}$ M DHT; or transfection media plus FBS for between 6 and 48 hours. Quantification of *Cldn11* expression is shown in Figure 3-21. Expression at 48 hours was greater than at the other timepoints; however the fold change was less than 1.5 for all 3 treatments. Analysis by 2-way AVOVA showed that expression was significantly different between time points (p=0.0005). Using the Bonferoni post-hoc test, specific differences were found between cells incubated in DHT at 24 and 48 hours (p=0.05), and cells incubated in DMSO at 48 hours compared to those at 16 or 24 hours (p= 0.05 and 0.01, respectively). Treatment of cells with DMSO or DHT at 48 hours or any of the 3 other time points did not result in expression of *Cldn11* that was significantly different from cells incubated with media (p=0.98).

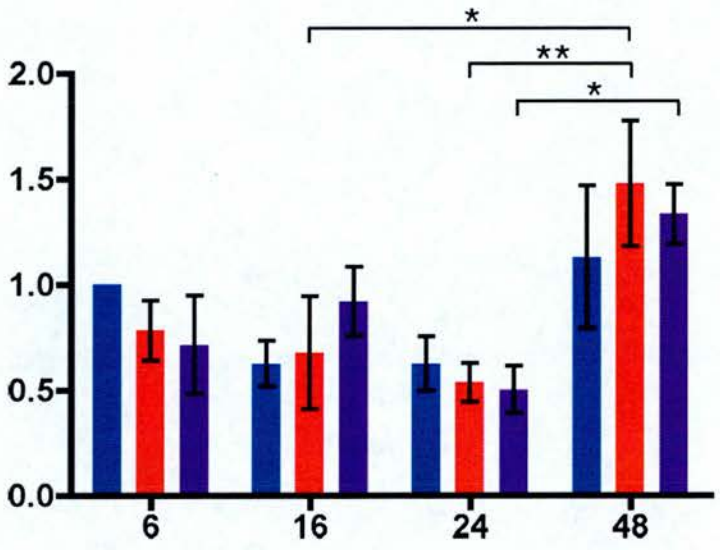


Figure 3-21: Expression of *claudin11* (*Cldn11*) mRNA in SK11 cells transfected with *mAR* quantified by Q-RT-PCR. Cells were incubated with media plus FBS (blue), DMSO (red) and $1x10^{-9}$ M DHT (purple). Expression was determined after incubation for 6, 16, 24 and 48 hours at 34°C. Expression is relative to that in cells incubated with media plus FBS for 6 hours; bars represent average expression ± SEM from 3 independent experiments. A 2-way ANOVA showed a statistically significant overall effect of time ($p= 0.0005$) but treatment with DMSO or DHT does not have a significant effect compared to media ($p= 0.98$). * = $p<0.05$ and ** = $p<0.01$.

3.3.4 Oestrogen responsiveness of SK11 cell line

3.3.4.1 ERβ mRNA expression assessed by RT-PCR

The expression of mouse *ERβ* in SK11 cells cultured at 34°C (lanes 1) and in cells differentiated by culture at 39°C for 48 hours (lanes 2) was detected by nested RT-PCR (Figure 3-22). Expression of *ERβ* was detected in cells incubated at both temperatures and did not appear to change when cells were cultured at 39°C; *ERβ* mRNA was also detected in adult mouse prostate, testis and kidney (lanes 3, 4, and 5 respectively).

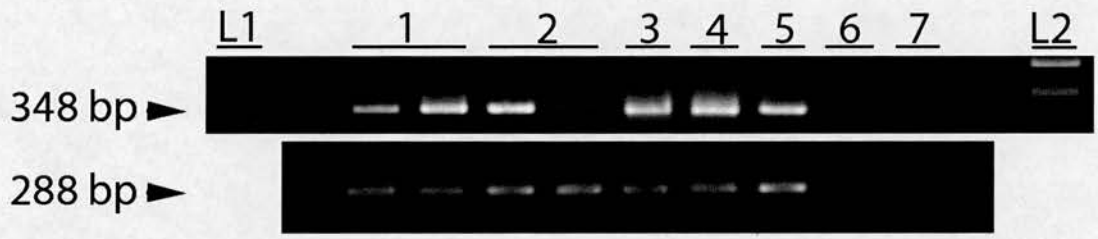


Figure 3-22: Detection of *ERβ* mRNA (348 bp) with RT-PCR in SK11 cells cultured at 34°C (lanes 1) or 39°C for 48 hours (lanes 2). Positive tissues: adult mouse prostate (lane 3), testis (lane 4), and kidney (lane 5) and negative controls: RT- testis sample (lane 6) and water (lane 7) were included. L1 is O'GeneRuler 1 kb ladder and L2 is Bioline Hyperladder I, band sizes (bp) are shown on the left of the figure. Expression of GAPDH mRNA (288 bp) in the same samples is included as a control.

3.3.4.2 ERE-luciferase reporter assay

SK11 cells were infected with an adenovirus containing an *ERE-Tk-luc* construct to determine whether they expressed a functional ERβ protein.

3.3.4.2.1 Time course

Luciferase protein expression was quantified (Figure 3-23) in SK11 cells infected with the viral ERE and incubated with media ± the antioestrogen ICI 182,780 (ICI), DMSO, E2 ($1x10^{-8}$ or $1x10^{-7}$ M) ± ICI or DES (10^{-8} M) for 12 to 48 hours. Luciferase expression was standardised to expression in cells incubated in media plus ICI for 12 hours and all changes were within 3-fold of this expression. Statistical analysis, by 2-way AVOVA, reveals that there was a significant difference in expression between time points ($p=0.005$) and that oestrogen treatment altered luciferase expression significantly ($p=0.01$). The increase in expression observed after incubation of cells with E2 was less dramatic than in response to DES, and similar to that of cells treated with the DMSO vehicle control. The impact of E2 on receptor gene expression was inhibited by inclusion of ICI with the oestrogen, reducing expression to a level similar to that in cells

incubated with media plus ICI. Luciferase expression was highest at 24 hours and effect of time was significant.

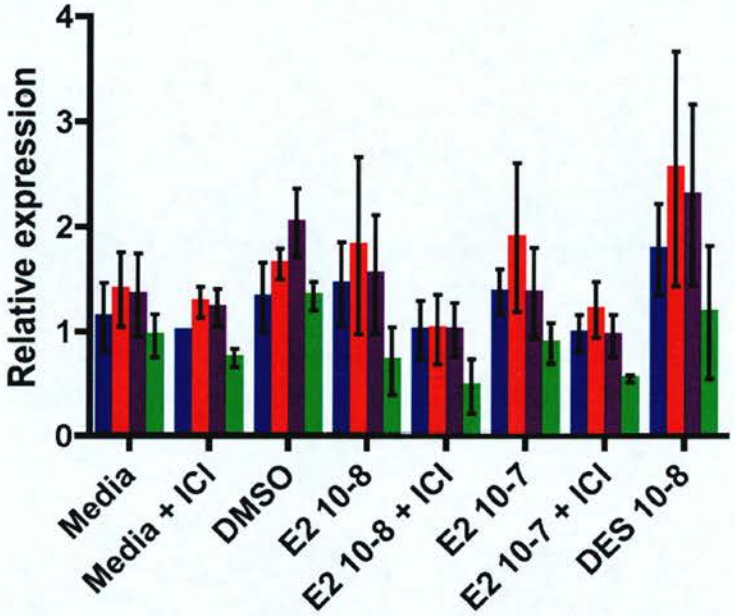


Figure 3-23: Luciferase expression in SK11 cells infected with *ERE-Tk-Luc* viral construct and treated with oestrogenic ligands in the presence or absence of ICI 182,780 (ICI). Cells were incubated for 12 (blue), 24 (red), 36 (purple) or 48 hours (green). Cells were cultured at 39°C for 48 hours prior to and for the duration of the experiment. All values are relative to Luc expression in SK11 cells cultured for 12 hours in media + ICI. Bars represent the mean Luc expression ± SEM based on n=3 experiments. A 2-way ANOVA demonstrated that both steroid treatments and duration of treatment significantly alter oestrogen response element activation (p= 0.01 and p= 0.005, respectively).

3.3.4.2.2 Ligands ± ICI

The cell treatments described in section 3.3.4.2.1 demonstrated that SK11 cells were capable of responding to oestrogens (E2 and DES), that this response was inhibited by ICI 182,780, and that 24 hours is a suitable time point at which to investigate oestrogen response in SK11 cells. The range of oestrogens and the concentrations at which they were used was broadened to identify the optimum oestrogen treatment. Figure 3-24 shows expression of luciferase protein in SK11 cells incubated for 24 hours with media, DMSO, ethanol, E2, Adiol and DES, all treatments were also duplicated with the

addition of 1 μM ICI. Expression was standardised to expression in cells incubated with media plus ICI, and over 2-fold expression was seen in some samples. Reporter gene activation was highest in cells treated with E2 at concentrations between $1x10^{-9}$ and $1x10^{-7}$ M, but all these ligands were able to increase the amount of luciferase. Expression of luciferase was inhibited by inclusion of ICI. Statistical analysis of the data by 2-way ANOVA shows that overall oestrogen treatment and ICI treatment significantly affected luciferase expression (both $p<0.0001$) and that these 2 factors had a significant interaction ($p=0.003$). Specifically the Bonferoni post-hoc analysis showed that luciferase expression was significantly greater in cells incubated with E2 at $1x10^{-9}$ M ($p<0.001$), $1x10^{-8}$ M ($p<0.01$) and $1x10^{-7}$ M ($p<0.05$) compared to cells incubated in media ± ICI. The inhibition of luciferase expression by ICI was significant in cells cultured with E2 at $1x10^{-9}$ M ($p<0.001$), $1x10^{-8}$ M ($p<0.01$) and $1x10^{-7}$ M ($p<0.05$) and Adiol at $1x10^{-7}$ M ($p<0.05$).

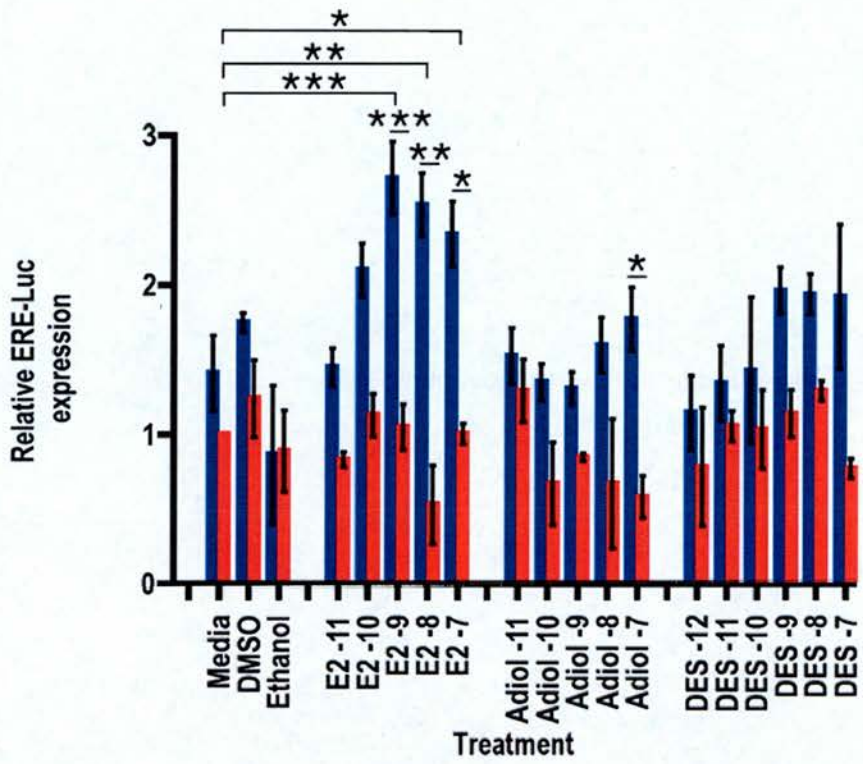


Figure 3-24: Luciferase expression in SK11 cells infected with *ERE-Tk-Luc* viral construct and treated with oestrogenic ligands in the absence (blue) or presence (red) of ICI 182,780 for 24 hours. Cells were cultured at 39°C for 48 hours prior to and for the duration of the experiment. All values are relative to Luc expression in SK11 cells cultured in media + ICI. Bars represent the mean Luc expression ± SEM, based on n=3 runs. Analysis by 2-way ANOVA shows that steroid and ICI treatment significantly affect activation of the oestrogen response element ($p=0.0009$ and $p<0.0001$ respectively). Steroid and ICI treatment interacted significantly to alter ERE activation ($p=0.003$). * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

3.4 Discussion

The SK11 cells are an immortalised SC line derived from 10 day old mouse testes, these cells have been assessed in terms of their gene and protein expression in a limited number of papers (Sneddon et al., 2005; Walther et al., 1996; Walther et al., 1997). In these papers it was reported that they expressed mRNA and protein for both AR and ERβ, and are able to stimulate expression of reporter gene constructs in response to androgens and oestrogens (Sneddon et al., 2005; Walther et al., 1996). To further our understanding of the SK11 cells' properties and to investigate expression of SC products with potential roles in control of spermatogenesis in response to steroid signals the studies discussed below were undertaken. The studies include further characterisation and comparison of gene and protein expression with that of immature and adult mouse SC, and assessment of androgen and oestrogen responsiveness of the SK11 cells.

3.4.1 Characterisation

Immunohistochemistry was performed on testes of day 10 and adult mice to assess the expression of AR, SGP-2 and Sdmg-1. These three proteins are expressed by mature SC and it was important to assess their expression in these and day 10 SC for comparison with the SK11 cells, which are derived from day 10 mice. The structure of the tubules within the day 10 testes was different to that of the adults, as described in the results section, briefly the tubules at day 10 lacked a distinct lumen and the nuclei of the SC were located away from the periphery of the tubules, approximately half way to the centre. This is in contrast to adult testes in which a distinct lumen is present and SC nuclei are located closer to the basement membrane of each tubule although the absolute shape and position of adult SC is stage-dependent. Sertoli cells express AR, SGP-2 and Sdmg-1 in both adult and day 10 mice, and whilst expression of Sdmg-1 is uniformly expressed in all tubules at both ages, the pattern of expression of AR and SGP-2 exhibit subtle differences between ages. In adult testes AR and SGP-2 expression in SC varies between tubules at different stages of the spermatogenic cycle, in day 10 tubules a full germ cell complement is not present, and SC in all tubules express AR and SGP-2 with

equal intensities. Expression of AR was also detected in nuclei of LC and PTM in the interstitium of testes at both ages. The stage specific AR expression observed in the adult SC, in addition to expression in LC and PTM cells, is consistent with the pattern of AR expression reported in previous rodent studies (Bremner et al., 1994; Tan et al., 2005; Zhou et al., 2002). The detection of AR expression in the d10 testis is in agreement with previous investigations in which AR expression was first detected in SC at 8 days post-partum (Tan et al., 2005). The expression of SGP-2 at both day 10 and in adult testes is in agreement with previous *in vivo* studies, as is the stage-dependent variation in expression in adult tubules (Tan et al., 2005).

To investigate the phenotype of the SK11 cells, the expression of mRNA and proteins characteristic of SC were assessed in SK11 cells cultured at permissive and non-permissive temperatures and compared to expression in mouse testes. Using semi-quantitative RT-PCR expression of *SGP-1* mRNA appeared to be higher in SK11 cells cultured at the non-permissive temperature over that in SK11 cells in permissive conditions. The expression of *SGP-2* mRNA exhibited a similar pattern with very weak expression in SK11 cells at the permissive temperature and strong expression in cells cultured at the non-permissive temperature. These results were extended by Taqman quantitative-PCR for *SGP-2*. Expression of *SGP-1* and *-2* were increased in mature testes compared to immature, although total SC mRNA was diluted by that of GCs expression of *SGP-1* and *-2* change in a similar manner when SC mature *in vivo* and when SK11 cells change from their proliferative to senescent 'differentiated' state. The increase in *SGP-2* mRNA in non-proliferative SK11 cells appeared to be paralleled by an increase in total protein detected by Western, however the change was not significant. In contrast expression of Sdmg-1 protein appeared similar in SK11 cells at 34°C and 39°C suggesting this protein is not affected by the differentiated states of the SC.

In the current experiments, assessment of AR mRNA and protein expression between cells cultured under permissive and non-permissive conditions did not detect any significant change in AR expression. The low *AR* mRNA in SK11 cells at both

permissive and non-permissive temperatures and low intensity of protein detected in Western analysis suggest the SK11 cells used in these studies expressed AR poorly. Immunohistochemistry did not detect any obvious difference in androgen receptor expression in SC nuclei of testes from day 10 and adult mice, with the only apparent difference being stage-specific expression in adults that was absent in the immature testes. Absence of changes in the amount of *AR* mRNA in SK11 cells between culture temperatures are in contrast to previous findings using SK11 cells where AR expression was increased in cell cultured under non-permissive conditions (Sneddon et al., 2005) and whilst it is difficult to compare experiments undertaken many years apart it is possible that extended use of this cell line and repeated passage may have resulted in selective loss of AR.

However the SK11 cells had clearly retained a number of characteristics associated with SC function. For example, compared to those at permissive temperatures, SK11 cells cultured under non-permissive conditions express higher *SGP-1*, *SGP-2*, and *Rhox5* mRNA levels, and have significantly increased protein expression of Rhox5. Previous studies have already investigated the expression of mRNAs including *SGP-1* and *-2* in SK11 cells. An increase in *SGP-2* mRNA expression under non-permissive conditions was first reported by Walther et al. (1996) and in the present study these results have been extended by Taqman quantitative RT-PCR and Western blotting. A previous study reported that *SGP-1* mRNA expression was similar at permissive and non-permissive temperatures (Sneddon et al., 2005) and although a marginal increase in *SGP-1* mRNA was detected using semi-quantitative PCR reactions, this was not confirmed using any other method so not too much emphasis should be placed on this result.

Data in this chapter demonstrate that SK11 cells express *Sdmg-1*, a gene first identified in fetal SC (Best et al., 2008), *Rhox5*, an androgen responsive gene expressed in SC and epididymis in the male reproductive tract (Lindsey and Wilkinson, 1996a; Lindsey and Wilkinson, 1996b), and mRNAs for *claudin3* and *11* whose proteins localise to the tight junctions between adjacent SC (Meng et al., 2005; Morita et al., 1999). Sdmg-1 protein

has been implicated in protein secretory pathways in fetal SC (Best et al., 2008), and interestingly a similar level of protein expression is maintained whether SK11 cells are cultured at 34°C or 39°C. Recent *in vitro* studies of Sdmg-1 used SK11 cells and reported that the protein localised to the endosomal compartment and demonstrated its role in secretory pathways (Best et al., 2008). Expression of *Rhox5* mRNA was significantly greater in SK11 cells incubated at 39°C compared to those at 34°C, and is in line with *Rhox5* mRNA first being detected in SC of mice 9 days post-partum. In adult mice Rhox5 expression is most intense in SC of stage VII and VIII tubules (Rao et al., 2003).

3.4.2 Androgen responsiveness of SK11 cells

Previous studies have demonstrated that SK11 cells at 34°C contain low levels of functional AR using a luciferase reporter gene driven by the proximal *Rhox5* promoter (Sneddon et al., 2005). However endogenous gene expression has not been explored previously. The impact of androgen (testosterone or DHT) treatment on *SGP-2*, *AR* and *Rhox5* mRNAs and on Rhox5 protein expression was assessed, in SK11 cells at both permissive and non-permissive conditions. Based on studies using *in vivo* models the expression of *SGP-2* mRNA was not expected to be androgen-responsive (McKinnell and Sharpe, 1995; Turner et al., 2001), and no change in expression in the cells was detected. SK11 cells at both permissive and non-permissive temperatures showed erratic expression of *AR* mRNA which was not increased by androgen treatment at any dose. From the evidence of previous studies, expression of AR protein appears to be androgen responsive, for example treatment of immature SC *in vitro* with testosterone or DHT results in increased androgen-binding (Verhoeven and Caillaeu, 1988). In rat testes subjected to androgen-depletion by EDS-treatment AR protein expression detected by immunohistochemistry is rapidly restored by testosterone ester treatment (Atanassova et al., 2006; Bremner et al., 1994; Turner et al., 2001). *Rhox5* has also been studied extensively as an androgen responsive gene expressed within SC *in vivo* and *in vitro* (Lindsey and Wilkinson, 1996b; Maclean et al., 2005), differential expression of *Rhox5*

between SCARKO and control mouse testes has been identified in recent studies (De Gendt et al., 2004; Denolet et al., 2006a). In this study treatment of SK11 cells with androgens in permissive and non-permissive conditions unexpectedly did not increase *Rhox5* mRNA or protein expression significantly. Lack of androgen response in SK11 cells may be due to the very low expression of *AR* mRNA discussed above, and as a consequence low functional AR protein levels that prevent AR mediated responses.

Due to the absence of androgen response in two well defined androgen-responsive genes, *AR* and *Rhox5*, the cause/s of the poor responsiveness of the SK11 cells was further investigated. SK11 cells were transfected with mouse *AR* plasmid to determine whether the SK11 cells were unable to support expression of functional AR protein. Transfected cells expressed significant quantities of *AR* mRNA and showed a dose-response between quantity of plasmid DNA introduced and amount of mRNA transcribed. The introduction of *AR* plasmid also resulted in translation of AR protein with dose-response seen between plasmid DNA introduced and amount of AR protein generated. These results show that the SK11 cells maintained the capacity to express the *AR* transgene and to generate AR protein. The AR protein also maintains a property of AR identified in previous studies, its structure is stabilised following androgen binding and is therefore more readily detected in techniques where it is identified using antibodies, such as Western blots and immunohistochemistry (Saunders et al., 1996; Zhou et al., 1995). Stabilisation of AR by androgens suggests that the protein expressed by SK11 cells is functional and this was further investigated using a reporter gene assay.

If a loss of AR expression was the only deficiency to androgen signalling in SK11 cells then transfection of cells with an appropriate quantity of mouse *AR* plasmid would allow increased expression of androgen-responsive genes in the presence of androgenic ligands. The capacity of SK11 cells transfected with the exogenous AR plasmid, to activate a well defined androgen-response element was tested by co-transfection of cells with the proximal *Rhox5* promoter linked to a *luciferase* reporter (Sneddon et al., 2005). This experiment showed that transfection of SK11 cells with mouse *AR* plasmid alone

was sufficient to enable the cells to stimulate the ARE containing promoter in the presence of androgens, demonstrating that SK11 cells were capable of expressing functional AR protein that can dimerise in the presence of androgens. The study also identified $1x10^{-9}$M DHT as the most effective dose for activation of the *Rhox5* response element, inducing significantly greater levels of reporter expression than untreated cells transfected with the same quantity of *mAR* plasmid. Stimulation of the same *Rhox5-luciferase* construct was reported by Sneddon et al to be stimulated by DHT and testosterone in SK11 cells, however the SK11 cells in that study did not require transfection with *AR* plasmid prior to stimulation (Sneddon et al., 2005). In the previous study, DHT concentrations of $1x10^{-7}$ and $1x10^{-6}$ M were required for maximum stimulation of the promoter; however in the present study a much lower concentration of $1x10^{-9}$ M resulted in optimal stimulation. A lower androgen concentration may be required as a consequence of higher concentrations of AR following transfection.

Despite the observation that transfected cells were capable of stimulating expression of *luciferase* linked directly to the *Rhox5* proximal promoter in response to androgen-treatment, they were unable to stimulate expression of endogenous *Rhox5* mRNA whose expression is reported to be controlled in SC through the same response elements (Sutton et al., 1998). Even over expression of AR protein using the highest quantities of *AR* plasmid did not enable the cells to regulate expression of *Rhox5* mRNA in response to androgens. The mechanism for increased *Rhox5* mRNA expression associated with increased expression of AR following transfection regardless of androgen stimulation is not clear. The amount of *Rhox5* mRNA in SK11 cells was also investigated between 6 and 48 hours after androgen treatment but no significant increase over controls was detected at any time point. Androgen treatment of *AR*-transfected SK11 cells also failed to result in consistent stimulation of mRNA expression of previously identified androgen-responsive tight junction proteins, *claudin3* and *claudin11* (Denolet et al., 2006a; Meng et al., 2005) compared with controls incubated without DHT.

The observation that SK11 cells incubated with DHT could stimulate an ARE-reporter construct but not the expression of three endogenous androgen-responsive genes was puzzling. The amount of AR detected in initial cultures of SK11 cells was expected to be very low, but replacement of AR using a cDNA construct failed to result in altered expression of Rhox5 in response to DHT. Secondly, in these cells derived from an immature testis, there appears to be a deficiency in signalling capacity that does not prevent expression from a promoter introduced by a plasmid vector, but is unable to stimulate expression from the same promoter in a native conformation in the gene promoter region. The loss of substantial AR expression and absence of endogenous androgen-response when AR was re-introduced may both be due to phenotypic drift in the SK11 cells due to the period of time over which the line has been maintained. They might also provide insight into the differences in androgen-responsiveness of immature versus adult SC.

3.4.3 Oestrogen responsiveness of SK11 cells

In addition to investigating androgen-response in SK11 cells, the capacity of these cells to respond to oestrogens was also explored. Expression of *ERβ* mRNA was detected in SK11 cells cultured at permissive and non-permissive temperatures; expression was modest - similar levels of expression were identified in mouse testes, prostate and kidney; and did not appear to be altered by culture conditions. The detection of *ERβ* mRNA in SK11 cells is in agreement with previous studies which have identified expression in the same cell-line (Sneddon et al., 2005), and with *in vivo* studies which detected *ERβ* mRNA and protein in SC of mice and rats (Saunders et al., 1997; van Pelt et al., 1999; Zhou et al., 2002). Identification of modest *ERβ* mRNA in the cells suggested they might maintain the capacity to respond to oestrogen-treatment, as described in a previous study (Sneddon et al., 2005).

The SK11 cells were able to induced expression of a luciferase reporter construct linked to three EREs in tandem in response to oestradiol (E2), diethylstilbestrol (DES) and 3β-

Adiol, at concentrations between 1 x 10^{-12} and 1 x 10^{-7} M, without the need for transfection with an *ERβ* plasmid. The expression of the reporter was specific for the action of oestrogens via ERβ because expression of the reporter was prevented by pre-treatment of cells with the oestrogen receptor antagonist, ICI 182,780 (Tremblay et al., 1997). Studies showed that the greatest responses to oestrogen treatment occurred 24 hours after the start of incubation, and that E2 was the most active of the 3 ligands. A dose of 1 x10^{-9} M was the optimum E2 concentration to use. Reporter gene expression was dose-responsive in the presence of all 3 ligands, and in all cases the responses were blocked by pre-incubation with ICI 182,780. In previous studies on SK11 cells' oestrogen response, maximal response to E2 treatment, at 1 x 10^{-7} M, caused reporter expression to rise to ~4.5 times that in untreated control cells (Sneddon et al., 2005), however in this study maximum reporter expression reached less than 3-fold. Unfortunately at this time no genes have been identified as candidates for oestrogen responsiveness in SC, and therefore it has not been possible to study endogenous gene expression in response to oestrogen treatment, as has been done for androgen responsive genes.

3.4.4 Conclusions

The SK11 SC line used in this study has been characterised and used in previous investigations, which have demonstrated changes in the cells phenotype when cultured at permissive (~34°C) and non-permissive temperatures (≥39°C) (Sneddon et al., 2005; Walther et al., 1996; Walther et al., 1997). In the course of the studies presented in this chapter the changes previously reported were confirmed (apart from the raised AR expression at 39°C), and the gene and protein expression of SK11 cells in permissive and non-permissive conditions were compared to the properties of immature day 10 mouse SC and adult mouse SC. The SK11 cells cultured in permissive conditions were found to be an approximation of immature SC, whilst non-permissive conditions shifted the SK11 cells towards a more differentiated, adult phenotype. SK11 cells have previously been shown to express *AR* and *ERβ* mRNA and protein, plus be capable of

responding to androgens and oestrogens (Sneddon et al., 2005). However in this study, only the oestrogen response as measured by its ability to activate a reporter construct could be demonstrated in unmanipulated SK11 cells. To achieve response to androgen treatments the SK11 cells required transfection with mouse *AR* plasmid so that substantial expression of *AR* mRNA and protein occurred. Transfected SK11 cells were able to stimulate expression of a luciferase reporter linked directly to the *Rhox5* promoter, but were still unable to stimulate endogenous androgen-responsive genes including *Rhox5*. As a consequence, it was not possible to use SK11 cells to confirm the putative androgen-responsiveness of the genes identified in previous array analysis (Denolet et al., 2006a). The causes of this deficient androgen response need to be investigated. Alternative models for investigating androgen-dependent effects on spermatogenesis via SC are required, examples include testis explants or *in vivo* experimental models, such as those discussed in the following chapters. Alternatively the loss of androgen-response may be indicative of a drift in cell phenotype caused by the length of time the cell-line has been maintained in culture. If this is the case, SK11 cells from earlier passages or another source may have retained androgen-responsiveness and could be used in future studies. The current SK11 cells still maintained the capacity to respond to oestrogens and mean further studies might aid in identification of oestrogen-responsive genes.

4 Targeting of gene expression to Sertoli cells using adenoviral constructs *in vivo*

4.1 Introduction

Each SC is associated with, and supports the development of, numerous germ cells so the SC population is many times smaller than that of the GC. Although SC are in a minority, they play an essential role in supporting the functional maturation of the GC through spermatogenesis, as described in chapter 1. For example, the necessity for androgen action, and therefore the expression of AR, in SC for successful spermatogenesis has been elucidated by De Gendt et al and Chang et al. (Chang et al., 2004; De Gendt et al., 2004) both of whom generated transgenic mice (SCARKO) with SC-specific ablation of AR. In adult SCARKO mice germ cells failed to mature normally and only 3% completed meiotic prophase to form round spermatids; the androgen-dependent processes in SC that support GC development have not yet been identified.

In chapter 3, an *in vitro* cell model, the SK11 line, was used to investigate the response of SC to both androgen and oestrogen signalling. Treatment of cells with steroid ligands was able to induce reporter expression regulated by androgen and oestrogen responsive elements. However, *in vitro* expression of endogenous *Rhox5*, an androgen responsive gene of SC, was not enhanced in response to androgens which was in contrast to what happens in SC within the seminiferous epithelium *in vivo* (De Gendt et al., 2004; Lindsey and Wilkinson, 1996b; Tan et al., 2005). This result, in addition to loss of steroid response in primary cultures of SC after 3-4 days (Denolet et al., 2006b), supports the view that SC require association with GC to maintain a fully differentiated phenotype (Syed and Hecht, 1997).

Studies have confirmed the importance of AR and ERβ in reproductive function, total ablation of AR results in a female phenotype including failure of testes to descend

(cryptochidism) (Lyon and Hawkes, 1970) and male mice lacking ERβ display abnormal mating behaviour (Temple et al., 2003). Cell specific knockouts of steroid receptors can be very valuable, and a method for knocking out AR specifically in SC using CRE-recombinase driven by the AMH promoter has been described (Chang et al., 2004; De Gendt et al., 2004). However this method requires that mice with a floxed allele of the gene of interest be available plus breeding and validation can be very time consuming and expensive as two lines of mice are required.

In place of establishing lines of transgenic mice using the Cre-lox method, due to the drawbacks outlined above alternative strategies were considered for ablating gene products specifically within SC. Introduction of constructs expressing short interfering RNA (siRNAs) specifically into SC as a way of reducing expression of target mRNAs was considered a viable option as a number of methods had been described that might allow us to achieve this. For example, expression of a RNAi could be achieved by introducing a construct containing a SC-specific promoter that regulated the expression of a short hairpin (shRNA) or microRNA (miRNA), into mouse pronuclei to which were then used to generate transgenic mice. Rao et al. (Rao et al., 2006) succeeded in reducing expression of WT-1 specifically in the SC of mice by this method using a transgene consisting of a hairpin-loop targeting WT-1, under the control of the proximal Rhox5 promoter. As an alternative to generating a transgenic line of mice expressing a RNAi construct we considered the potential that it could be introduced directly into the SC by infection of the cells with an adenoviral vector containing an appropriate construct (e.g. Rhox5 promoter driven miRNA). Previous studies have reported the use of adenoviral vectors to introduce transgenes, including those expressing *CREB*, *LacZ* or *Steel factor* (*kit ligand*), into SC both *in vitro* and *in vivo* resulting in efficient expression (Blanchard and Boekelheide, 1997; Kanatsu-Shinohara et al., 2002; Scobey et al., 2001). In co-cultures of SC and GC, as well as *in vivo* studies in whole testes, GC have been shown to be resistant to infection by adenoviruses even at high concentrations (Blanchard and Boekelheide, 1997; Scobey et al., 2001). In study outlined in this study expression of the viral transgene was associated with specific stages at different time

points after infection, suggesting that adenoviral vectors predominantly infect SC at stages II-IV in rats (Blanchard and Boekelheide, 1997). It has been reported that SC-specific expression of transgenes introduced using adenoviral vectors was achieved without significant immune response when the vector was introduced by injection via the efferent ductules/rete testis or by a direct intra-tubular route. Limited immune cell (lymphocytes, plasma cells or myeloid cells) infiltration of injected tissues was identified when routes via the rete testis were used (Blanchard and Boekelheide, 1997; Kanatsu-Shinohara et al., 2002; Scobey et al., 2001). In the present study intra-tubular injection of adenoviral vectors via the efferent ductules was used both because of reports of only limit immune response and the presence of an established method of efferent duct injection previously used for GC-transplantation studies within the laboratory.

4.1.1 Aims

To establish methods for infection of mouse SC with adenoviral vectors expressing reporter constructs both *in vitro* and *in vivo*. In addition, to determine whether infection with adenoviral constructs has any impact on testicular function and to use adenoviral vectors to introduce RNAi constructs specifically into SC *in vivo*.

4.2 Materials and methods

4.2.1 Virus preparation

Three recombinant adenoviral constructs were used; these expressed *β-galactosidase*, *green fluorescent protein* (*GFP*), or had no insert. High titre stocks ($1.3x10^{10}$ – $1.66x10^{10}$ plaque forming units (pfu)/ml) of viral particles were prepared for each construct as described in section 2.6. Expression of *β-galactosidase* was controlled by a cytomegalovirus immediate early (*CMV IE*) promoter (*RAD35*) (Wilkinson and Akrigg, 1992) and the negative control contained a *CMV IE* promoter but no downstream reporter (*RAD60*); both constructs were kind gifts from Professor AH Baker (British Heart Foundation, Glasgow Cardiovascular Research Centre, Glasgow, UK). The *GFP* construct was an *Ad-CMV-GFP* construct purchased from Vector Biolabs, (Philadelphia, PA, USA).

4.2.2 *In vitro* infection

SK11 cells were cultured at 34°C and 39°C, 5% CO_2, in 'complete' phenol-red free media, as described in section 2.2. Cells were seeded at $1x10^5$ cells/well in 12-well plates for 48 hours prior to infection with the adenoviral *β-galactosidase*, *GFP* or *RAD60* constructs. For the initial titration experiments expression of reporter constructs and proportions of live and dead cells were determined using the Ad-β-galactosidase construct at MOIs between 10 and 250. Thereafter the three viral constructs were used at MOIs within the range 50-100. When infecting cells with any of the viral constructs the procedure was identical. All media was removed from the wells and replaced with 400 µl of fresh phenol red-free media, which was just enough to cover the surface of the well. The viral stocks were diluted in phenol red-free media to deliver the appropriate number of plaque forming units for each MOI (multiplicity of infection; number of infective viral particles / cell) in a volume of 50 µl/well. The cells were cultured with the virus for 4 hours at 37°C in 5% CO_2. The media and virus were removed and replaced with phenol red-free media and cells were cultured for a further 96 hours, in

5% CO_2, at 34°C or 39°C. After 96 hours incubation expression of GFP or β-galactosidase protein expression was determined and images collected as below.

4.2.2.1 Time Course of Infection

In addition to the studies above SK11 cells were also infected for 4, 6, 8, 12, 24 or 48 hours with the GFP adenoviral construct. After infection the cells were used in RNA extractions (section 2.3.1), and Q-RT-PCR was carried out for *interleukins -6* (section 4.2.6). RNA from SK11 cells not infected with viral particles was collected at each time point in parallel with the samples from the infected cells, and were used as controls. The media in which the cells were incubated was collected at each time point and used in ELISAs for inteleukins -6 (section 4.2.9).

4.2.2.2 β-galactosidase staining

Media was removed, cells were washed with PBS and 1ml LacZ fixation solution (2% formaldehyde (VWR) and 0.2% glutaraldehyde (VWR) diluted in sterile PBS) was added to each well for 5 minutes at room temperature. The fixed cells were stained with 1 ml/well of staining solution containing X-gal (Invitrogen, Paisley, UK) overnight at 37°C (see below for constituents). Expression of the LacZ gene by infected cells produces the enzyme β-galactosidase which catalyses the production of 4-chloro-3-brom-indigo, a blue precipitate, from the X-gal present in the staining solution (Figure 4-1). Staining solution was replaced with PBS the following day to allow staining to be viewed and photographed (section 4.2.8)

Figure 4-1: β-galactosidase reaction. Taken from http://www.fermentas.com/catalog/ reagents/x-gal.htm.

X-gal staining solution	0.5 M NaH_2PO_4	23 ml
	0.5 M Na_2HPO_4	77 ml
	Potassium ferrocyanide	1.06 g
	Potassium ferricyanide	0.82 g
	1% deoxycholate	5 ml
	2% nonidet P-400	5 ml
	1 M $MgCl_2$	1 ml

The solution was made up to final volume of 500 ml with distilled water. X-gal was added to the stain solution immediately prior to use; 1 mg X-gal dissolved in dimethylformamide (100 mg/ml, BDH) was added per millilitre of the solution above.

4.2.3 Intra-testicular injection

4.2.3.1 Preparation of viral solutions

The three viral constructs (section 4.2.1) were diluted in sterile saline and 20% (v/v) Trypan blue (0.08% w/v, Sigma) to a concentration equivalent to between $4x10^8$ and $1x10^5$ pfu per 50 µl of solution. Table 4-1 shows the various doses prepared for each viral construct:

Table 4-1: Doses of each adenoviral construct injected into mouse testes.

Construct	pfu/50µl			
	$4x10^8$	$1x10^7$	$1x10^6$	$1x10^5$
GFP	X	X	X	X
β-galactosidase	-	-	X	-
RAD60	-	-	-	X

4.2.3.2 Surgery

Animal experimentation was performed under the conditions described in chapter 2 (section 2.1.1.). Male mice on a *C57 BL/6* background were used throughout the study. The procedures for intra-testicular injection were essentially those of Ogawa et al. (Ogawa et al., 1997), and the surgical exposure of the reproductive tract and canulation of the efferent ducts were performed by Dr. M. Paterson. Surgery was performed under aseptic conditions with anaesthesia and analgesia. Deep anaesthesia was induced with ketamine/medetomidine (intraperitoneal injections), analgesia was ensured by administration of buprenorphine (subcutaneous injection) at the time of surgery.

An area of the abdomen over the left testis was shaved and sterilised with chlorohexidine (Sigma) before a small incision was made in the abdomen and muscle wall. The left testis and epidermis were withdrawn through the incision and manipulated on a sterile swab, the testis and swab were kept moist with sterile saline at all times. The efferent ducts were identified under a dissecting microscope and isolated from surrounding connective tissue. A small incision was made in the efferent duct close to the rete testis with a hypodermic needle providing access to canulate the duct with a glass needle and glass micro-needle syringe. The needles were produced in-house and had a diameter of 5 μm at the tip and were ground to a tapered point to aid insertion into the duct. The virus/dye solution (section 4.2.3.1) was gently injected into the seminiferous tubules up to a total volume of 50 μl and penetration of the blue dye through the tubules was monitored. The testis and epididymis were returned to the abdominal cavity and the muscle and skin layers were closed with internal and external vicryl (Ethicon, Livingston, UK) sutures. Following surgery the sedative effect of medetomidine was reversed with atipamezole to quickly revive the mice and they were kept warm and observed frequently in the post-operative period.

The right testis in each of the animal on which surgery was performed remained undisturbed as a paired-control for the injected testis. Both testes were recovered from each animal 2, 4 or 7 days after surgery; recovery, fixation, and preparation for

immunohistochemistry were performed by the methods described in sections 2.1.3 and 2.5.

4.2.4 Tunel assay for detection of apoptotic cells

Sections were stained using the in situ apoptosis detection (Tunel) assay to detect single stranded DNA consistent with DNA breaks (Gavrieli et al., 1992) as follows. Sections were dewaxed and rehydrated by standard methods (section 2.5.1) and blocked in 3% hydrogen peroxide in methanol (section 2.5.2.2) for 30 minutes. Sections were washed twice, for 5 minutes each, in PBS then placed on ice-cold trays to cool before addition of 50 µl/section of TdT/Dig-11-dUTP reaction mixture. The reaction mixture consists of 400 U/ml terminal d-transferase (TdT, Roche) and 5 µl/ml 1mM digoxigenin-11-deoxy-uridine-5'-triphosphate (Dig-11-dUTP, Roche) diluted in reaction buffer (30 mM TRIS/HCl pH7.2 (Sigma), 140 mM sodium cacodylate (VWR international) and 1.5 mM $CoCl_2$ (VWR international) made up in dH_2O. Reaction buffer was sealed on the slide under a GelBond (Cambrex, Rockland, ME, USA) coverslip with cow gum/hexane, and heated to 37°C for 30 minutes on a Hybaid Omnislide to enable incorporation of dUTP by the TdT enzyme. The slides were washed twice for 5 minutes in PBS to remove the reaction mixture, and then blocked with normal rabbit serum (NRS, dissolved 1 in 5 in PBS) for 10 minutes at room temperature in humidified conditions. Blocking buffer was replaced with sheep anti-Dig primary antibody (Roche) diluted 1:100 in NRS/PBS, which was incubated for 90 minutes at room temperature in humidified conditions. Sections were washed twice with PBS, 5 minutes each, and then incubated for 30 minutes with rabbit anti-sheep biotinylated antibody (Vector, Peterbourgh, UK) diluted in NRS/TBS (1 in 5) at room temperature. After incubation with the secondary antibody slides were washed in TBS twice for 5 minutes. The Tunel staining was visualised with ABC-HRP and DAB and sections were counterstained, dehydrated and mounted as described sections 2.5.2.7 and 2.5.2.8.

4.2.5 Haemoxylin and Eosin (H&E)

Sections from both testes (one control and the other infected) of mice that underwent intra-testicular injection were stained with H&E to reveal the general histology of the testes as follows. Sections were dewaxed and rehydrated in xylene and a descending series of graded ethanol baths as for immunohistochemistry (section 2.5.2). Rehydrated sections were washed in haematoxylin for 5 minutes, staining was differentiated by washing in acid-alcohol for up to 20 seconds to preferentially withdraw haematoxylin from the cytoplasm and the stain was precipitated in the nuclei with Scott's tap water for 20 seconds. The cytoplasm of the sections was visualised by staining with eosin for 20 seconds. Between each stage of staining sections were washed in tap water. Once stained with haematoxylin and eosin the sections were dehydrated in graded ethanol baths and xylene and were mounted under glass coverslips with pertex, as described in section 2.5.2.8.

4.2.6 Q-RT-PCR

Synthesis of cDNA from RNA recovered from SK11 cells infected with virus (section 4.2.2.1) was performed with random hexamer primers (section 2.3.3.1), and the TaqMan PCR reaction for IL-6 was undertaken using the Roche Universal Human Probe Library™ method (section 2.3.3.4). Results were analysed using uninfected SK11 cells, cultured in parallel with the infected cells, as the control groups. The expression level in uninfected cells at the 4-hour time point was taken as equal to 1. The primers and probe used are shown in Table 4-2.

Table 4-2: Primer and probe sequences used to quantify interleukin expression in SK11 cells.

Gene	Forward Primer 5' – 3'	Reverse Primer 5' – 3'	Probe (Probe number)
Interleukin-6	GCTACCAAACTG GATATAATCAGGA	CCAGGTAGCTAT GGTACTCCAGAA	TTCCTCTG (#6)

4.2.7 Immunohistochemistry

Immunohistochemistry was performed on testis sections from virally infected mice using antibodies raised against GFP, Caspase-3, HIF-1α, smooth muscle actin, SDMG-1 and junctional proteins. The procedures were as described in section 2.5. Caspase-3 and SDMG-1 were visualised with DAB staining using the standard protocol (section 2.5.2), GFP and HIF-1α using the Bond-X protocol and junctional proteins MPO, CD68 and SMA by double fluorescent staining as described in section 2.5.3. The antibodies and conditions used are shown in Table 4-3.

Table 4-3: Primary antibodies for immunohistochemistry.

Antigen	Dilution	Species raised in	Source
DAB			
caspase-3	1:200	Rabbit	Cell Signalling
GFP	1:1000	Rabbit	Molecular probes
HIF-1α	1:200	Rabbit	Abcam
Sdmg-1	1:1000	Rabbit	Gift from Ian Adams (MRC HGU, Edinburgh)
Fluorescent			
CD68	1:50	mouse	DAKO
Cx43	1:100	rabbit	Zymed
espin	1:30	mouse	Transduction laboratories
MPO	pre-diluted	rabbit	Abcam
SMA	1:500	mouse	Sigma

4.2.8 Imaging

Sections stained with DAB using the Bond-X machine, stained in the Tunel assay or after staining with H&E were all visualised on an Olympus Provis microscope (Olympus, Optical Co., London, UK) and images were captured with a Canon EOS 30D (Canon Europe, Amsterdam) (section 2.5.4.1).

The expression of proteins introduced via transient transfection or adenoviral infection into cell lines was also examined. Expression of LacZ was determined by staining for β-galactosidase enzyme activity (section 4.2.2.2) which was visualised using an Olympus CK2 inverted microscope (Olympus, Middlesex, UK) and Nikon D1 digital SLR camera (Nikon, Kingston-upon-Thames, Surrey, UK). *In vitro* expression of GFP (section 4.2.2) was visualised on an Axiovert 200M microscope (Zeiss). The appearance and numbers of the cells following infections was determined using an inverted Olympus CK2 microscope and photographic images were taken with a Nikon D1 digital SLR.

4.2.9 Interleukin ELISA

Media from cultures of SK11 cells infected with 100 MOI of the GFP adenoviral construct were used in an ELISA for IL-6 (R&D systems). The assay was performed as described in the manufacturer's protocol unless stated otherwise. The procedure was as follows. A series of eight interleukin standard solutions, with concentrations between 0 pg/ml and 500 pg/ml, were prepared by serial dilution and a control sample with a concentration within a known range was diluted as instructed. The wells of the ELISA plates are pre-coated with the appropriate anti-interleukin antibody, and 50 µl of assay diluent was added to each well. A volume of sample (100 µl), control (50 µl) or standard solution (50 µl) were added to the assay diluent in each well, with control and standards added to the plate in duplicate. The solutions were gently mixed and incubated at room temperature for 2 hours. The wells were thoroughly washed five times with wash buffer before addition of 100 µl mouse IL-6 conjugate that binds the interleukins attached to the coated wells. The conjugate was incubated on the plate for 2 hours at room temperature then the wells were washed five times. Each well was

incubated with 100 µl of Substrate solution for 30 minutes at room temperature whilst protected from light, after which 100 µl of Stop solution was added to the Substrate solution. The optical density of the solutions in each well was measured at 450 nm on a Labsystem Multiskan EX, with a second reading at 540 nm used to correct for imperfections in the plate. A standard curve of interleukin concentration against optical density was plotted and the accuracy of the ELISA confirmed by accurately predicting the interleukin concentration in the control sample within the range stated. The concentration of interleukin within each sample was calculated from its optical density.

4.3 Results

4.3.1 Impact of viral infection on Sertoli cells *in vitro*

4.3.1.1 Viral infection at different MOI

Viral infection of SK11 cells cultured at 34°C was performed at MOI between 10 and 250, with adenoviral particles containing a *LacZ* construct. The *LacZ* expression detected by staining for β-galactosidase activity is shown in Figure 4-2. No β-galactosidase was detected in uninfected cells, after infection with 10 MOI β-galactosidase expression was very weak. Intense uniform β-galactosidase activity was detected after infection with 25 and 50 MOI. The amount of cell detachment, associated with disturbance of the cell and death, witnessed in these wells was negligible as complete coverage of the wells' surfaces was still observed. At the highest MOIs used (100 and 250) although intense reaction product was detected there was a dramatic loss of cell from the surface of each well (Figure 4-2, panels E and F).

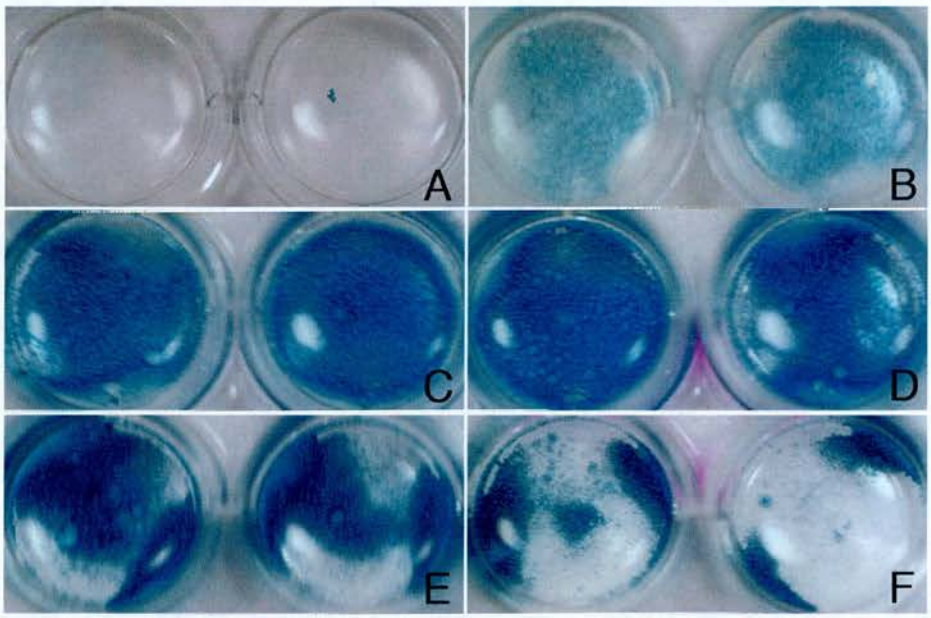


Figure 4-2: Cell survival of SK11 cells infected with a *LacZ* adenoviral construct at MOIs between 0 and 250. SK11 cells were cultured at 34°C for 96 hours following infection before expression of β-galactosidase was visualised by X-gal staining. SK11 cells were infected at the following MOIs: 0 (A), 10 (B), 25 (C), 50 (D), 100 (E), and 250 (F).

4.3.1.2 Comparison of LacZ and GFP viral constructs

SK11 cells cultured at 34°C were infected with adenoviral *GFP* and *LacZ* constructs for 4 hours and incubated for a further 96 hours before protein expression was assessed by fluorescent microscopy or X-gal staining, respectively (Figure 4-3). Cells were infected with MOIs between 0 and 100, no endogenous expression was seen at 0 MOI. Expression of reporter constructs was detected but low at 50 MOI for both constructs, expression was greater for both constructs at higher MOI. The expression of GFP peaked at 85-100 MOI with a large rise in expression seen between 75 and 85 MOI. Staining for *LacZ* reporter expression was present in a greater proportion of cells at 60 and 75 MOI than at the same MOI in GFP infected cells, and the peak β-galactosidase expression was observed following infection at 85 and 100 MOI. Cell density observed

following infection with the *LacZ* construct at 100 MOI was reduced compared to 85 MOI. The effect of adenoviral infection on cell survival is described in section 4.3.1.3.

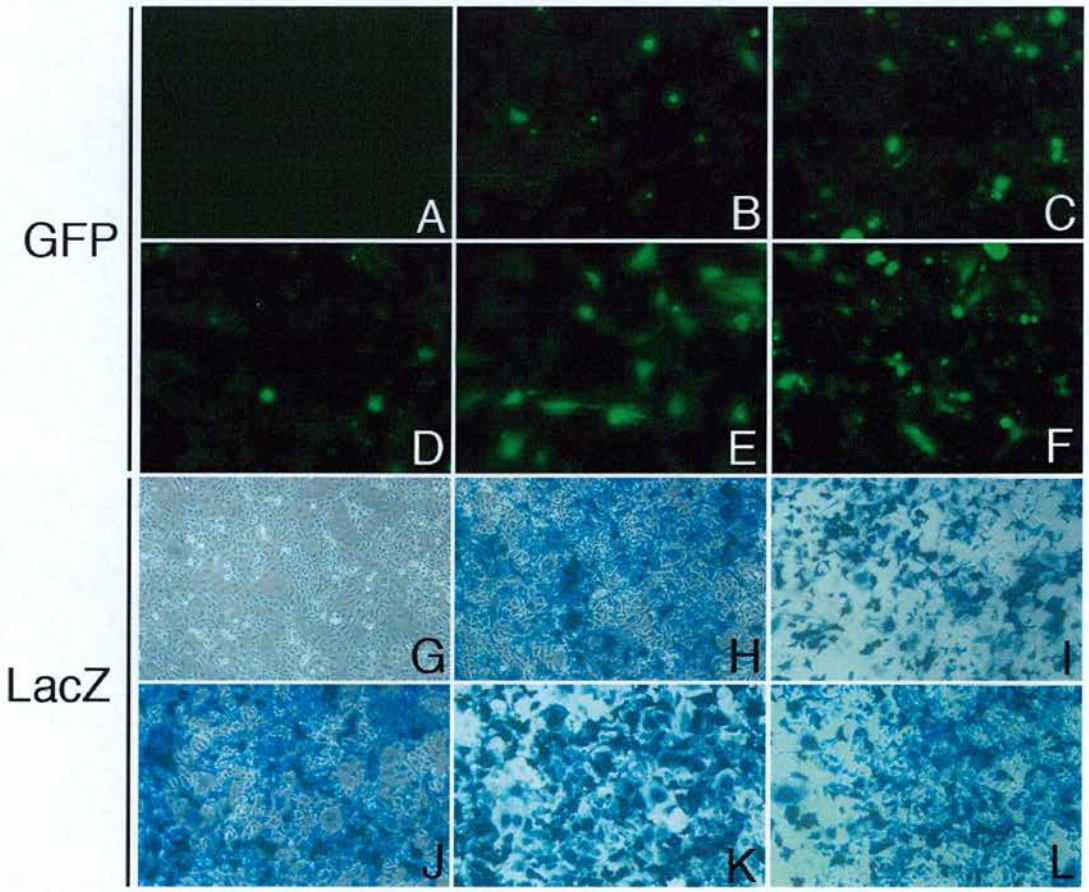


Figure 4-3: Expression of GFP (panels A-F) or β-galactosidase (panels G-L) reporter constructs following incubation with adenovirus constructs. SK11 cells were incubated at MOI 0 (panel A and G), 50 (panels B and H), 60 (panels C and I), 75 (panels D and J), 85 (panels E and K), and 100 (panels F and L) for 4 hours, and reporter expression was visualised after 96 hours.

4.3.1.3 Impact on cell survival

Adenoviral particles containing *LacZ* or *GFP* constructs, or the *RAD60* virus which had no insert, each used to infect SK11 cells at between 50 and 100 MOI with each viral

construct. Cell densities 96 hours after infection are shown in Figure 4-4. There was a decline in the number of cells present with increasing MOI for all three viral constructs, and there was not a significant difference in cell loss between constructs at each MOI. In spite of the results in section 4.3.1.1 there was cell loss between the control (0 MOI) and infection at 50 MOI, but the loss was less acute than at 75 and 100 MOI.

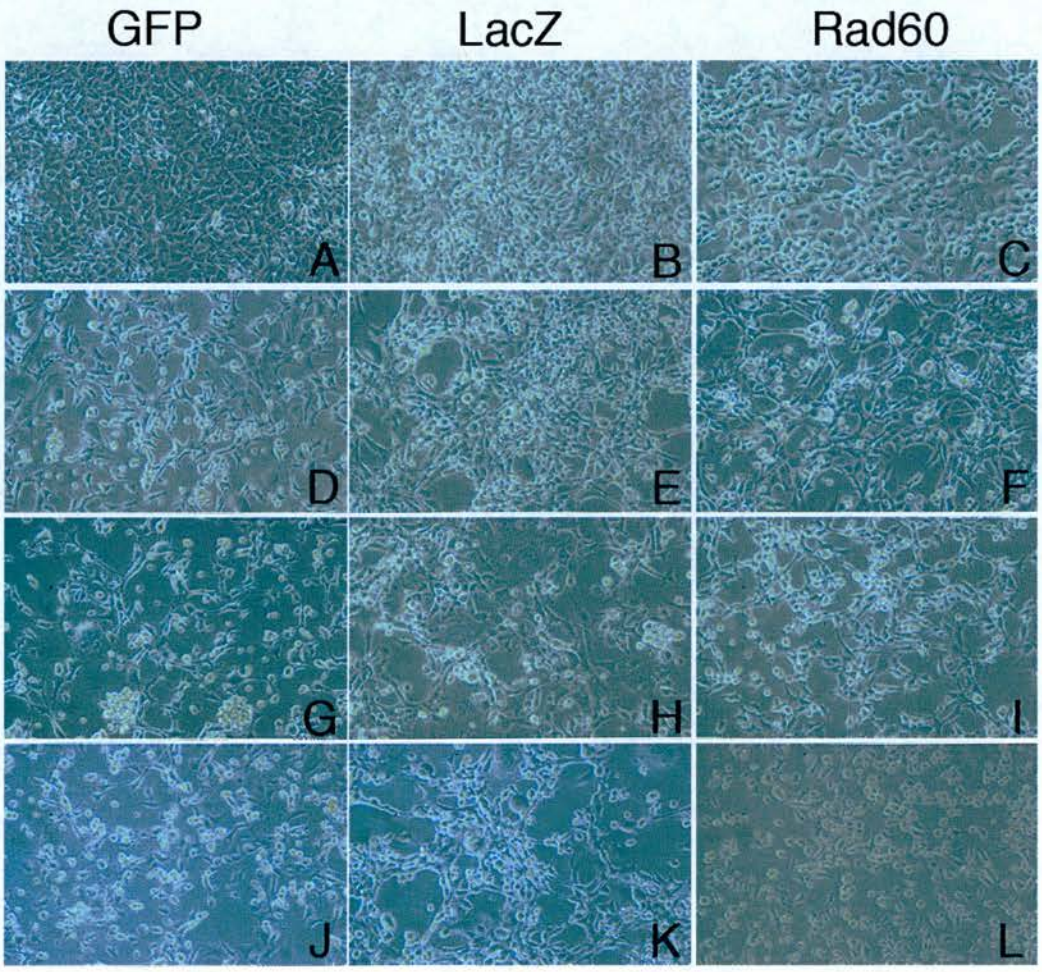


Figure 4-4: Densities of SK11 cells 96 hours after infection with GFP (A, D, G, and J), LacZ (B, E, H, and K), or RAD60 (C, F, I, and L) adenoviral constructs at a range of MOIs. SK11 cells were infected at MOIs of 0 (A-C), 50 (D-F), 75 (G-I), and 100 (J-L). Images are at 20x magnification.

4.3.1.4 IL-6 production

The capacity of SK11 cells to produce interleukin-6 in response to infection by the *GFP* adenoviral construct was assessed. SK11 cells were cultured at 34°C and 39°C for 4 to 48 hours with 100 MOI adenovirus; IL-6 protein secreted into the culture media was measured by ELISA (Figure 4-6), and expression of *IL-6* mRNA (Figure 4-5) was assessed by Q-RT-PCR. The amount of IL-6 protein progressively accumulated in the culture media over 48 hours and SK11 cells cultured at 39°C produced significantly more IL-6 than cells cultured at 34°C. Despite the difference in IL-6 protein expression between temperatures, infection by adenovirus was not associated with an increased secretion of IL-6 at either temperature.

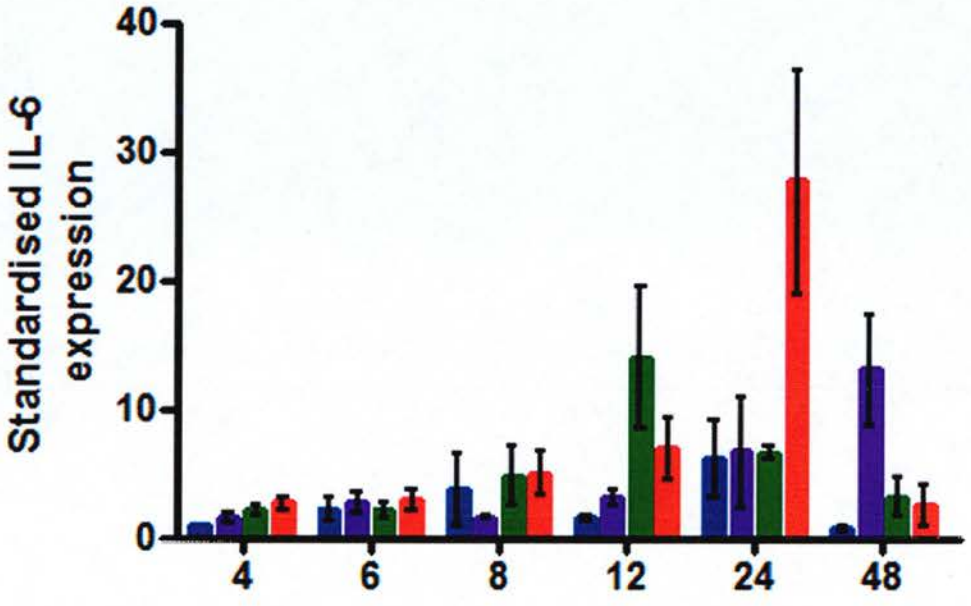


Figure 4-5: Expression of *IL-6* mRNA following culture of SK11 cells in the absence (blue and green bars) and presence (red and purple bars) of GFP-adenoviral constructs, at MOI 100. Cells were cultured for 4-48 hrs at 34°C (blue and purple bars) and 39°C (green and red bars). Expression was standardised to that in SK11 cells cultured at 34°C for 4 hours in the absence of adenovirus. Two-way ANOVA demonstrated that time and treatment both significantly affected *IL-6* expression (p=0.0003 and p=0.02 respectively), these two factors also interact significantly (p=0.001).

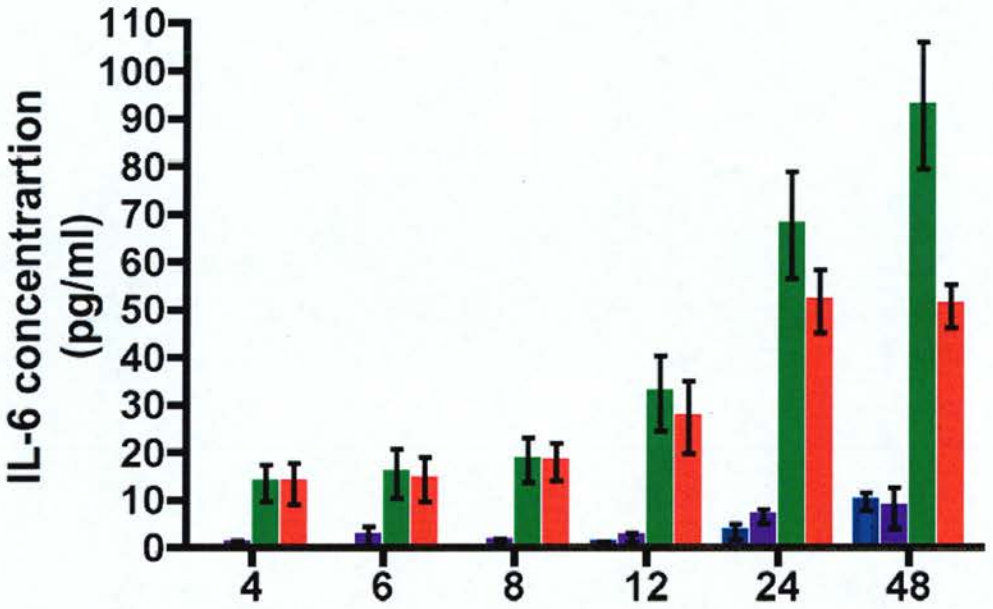


Figure 4-6: Expression of IL-6 following culture of SK11 cells in the absence (blue and green bars) and presence (red and purple bars) of *GFP*-adenoviral constructs, at MOI 100. Cells were cultured for 4-48 hrs at 34°C (blue and purple bars) and 39°C (green and red bars). IL-6 secretion was quantified through an ELISA performed on culture media. Two-way ANOVA demonstrated that time and treatment (plus interaction between these 2 factors) both significantly ($p < 0.0001$) affected IL-6 expression.

4.3.2 Impact of adenoviral infection on Sertoli cell function *in vivo*

4.3.2.1 Immunolocalisation of GFP demonstrates Sertoli cell specific infection

Expression of the GFP following intra-testicular injection was detected by immunohistochemistry Figure 4-7. Expression was Sertoli cell-specific and the intensity of immunostaining was influenced by the quantity of plaque forming viral particles introduced into the testis. Expression in testes injected with $4x10^8$ pfu (panels A and B) was the most intense, but cell-specific expression was hard to determine due to the disrupted tubule structure. In testes infected with $1x10^7$ pfu per testis (panels B and C) GFP staining was less intense than in panels A or B, but was more obviously SC-

specific. In testes infected with $1x10^6$ pfu per testis GFP expression was SC-specific but much weaker and the intensity of staining was reduced even further at $1x10^5$ pfu per testis. Uninfected testes were immunonegative (panel I).

Within the injected testes only a limited number of tubules were immunopositive for GFP expression, even at the two highest doses of virus. Expression of GFP was associated with disturbance of tubule architecture, but many apparently damaged tubules did not exhibit staining for GFP. Even when disrupted tubules were in close proximity to a heavily stained tubule they did not necessarily express GFP (panels C and D); and at lower viral doses despite damage to the tubules, infection was often not sufficient for detectable GFP expression within the testis (panels G and H).

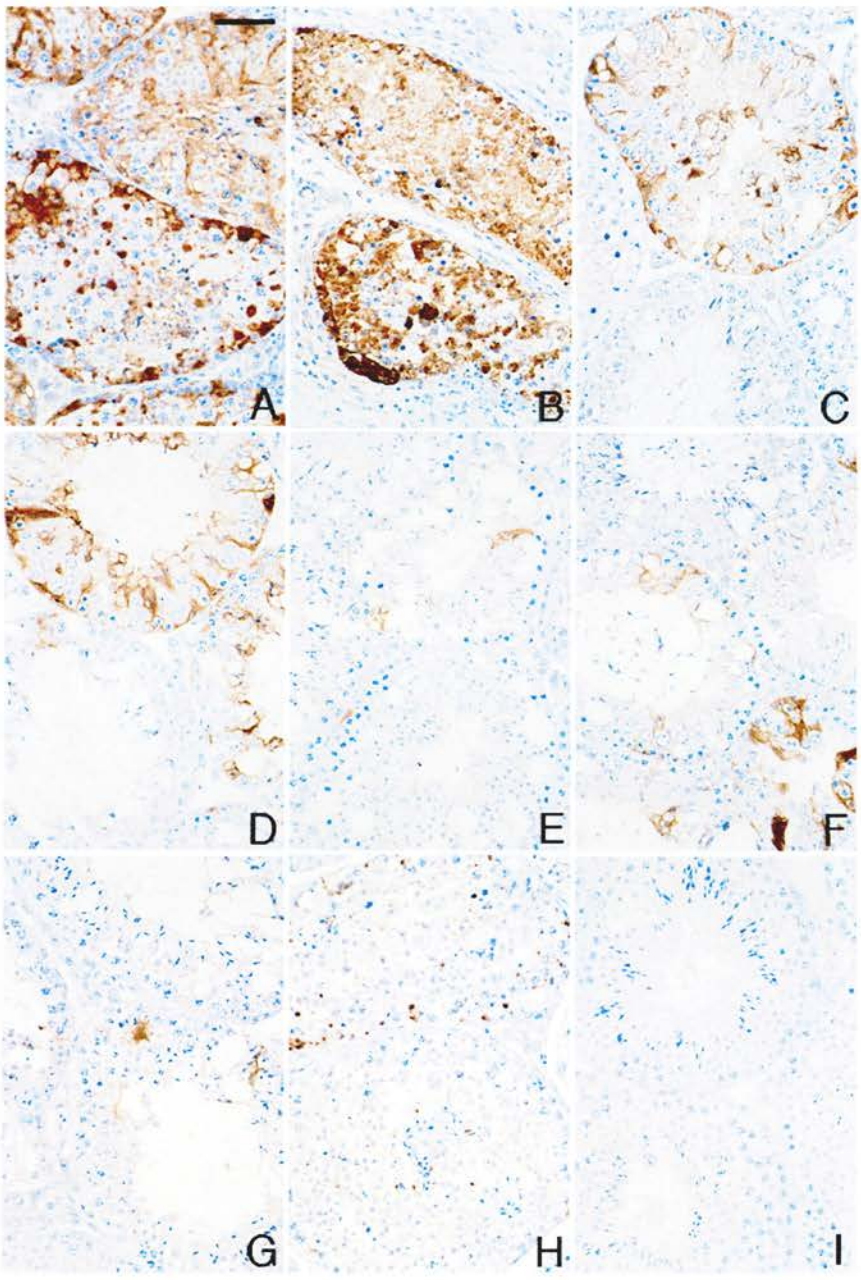


Figure 4-7: Immunological staining of GFP expression in mouse testes infected with a *GFP* adenoviral construct at a range of doses. Seminiferous tubules of the testes were specifically infected by intra-testicular injection. Testes were collected 4 days after infection except for those in panel A (3 days) and panel B (7 days). The doses of virus given per testis were: $4x10^8$ pfu (A and B), $1x10^7$ pfu (C and D), $1x10^6$ pfu (E and F), $1x10^5$ pfu (G and H). The untreated contralateral testis from the same animal as the testis in panel A is shown as a control in panel I. Images are 40x magnification and the scale bar represents 50 μm.

4.3.2.2 Impact of viral infection on testicular architecture

4.3.2.2.1 Haematoxylin and eosin

Sections from the testes infected with between $1x10^4$ and $4x10^8$ pfu per testis were stained with H&E and are shown in Figure 4-8 (x20) and Figure 4-9 (x100). The structure of the tubules in sections from testes infected with $4x10^8$ pfu/testis was severely disrupted at both 3 days (A) and 7 days (B) post infection notably, the lumen was absent and GCs were displaced or absent. At both time points, elongated spermatids were lost and cells were present in the interstial and seminiferous tubules that resembled immune cells, specifically neutrophils and lymphocytes (marked with arrows in panel B of both figures). Disruption of structures was significantly worse in the testis collected 7 days (panel B) after infection and identification of GCs was impossible due to the scale of disruption and invasion.

In sections from testes infected with lower viral concentrations ($1x10^5$ - $1x10^7$ pfu/testis) overall tubular architecture was present, a lumen was visible and GCs could be identified. However within the seminiferous epithelium 'vacuoles' appeared (indicated by arrows in panels C-H in Figure 4-8 and Figure 4-9). Preliminary analysis suggests that a significant proportion of the population of pachytene spermatocytes were missing from the tubules and that the spaces created by this GC loss accounted for the 'vacuoles'.

At the three lowest doses of adenovirus not all seminiferous tubules were disrupted within the infected testes. Both disrupted and normal tubules were found in close proximity within sections, the normal tubules were indistinguishable from tubules of uninfected testes.

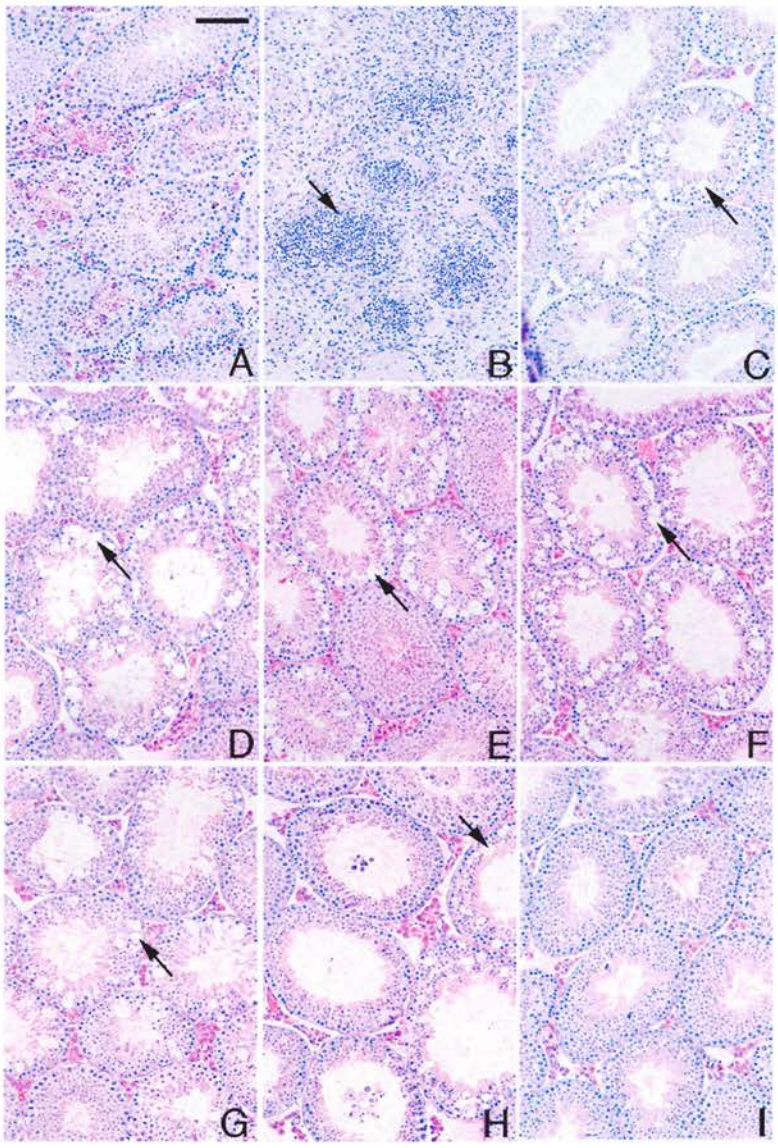


Figure 4-8: Haemoxylin and Eosin staining of adult mouse testes infected with a *GFP* adenoviral construct at a range of doses. Seminiferous tubules of the testes were specifically infected by intra-testicular injection. Testes were collected 4 days after infection except for those in panels A and I (3 days) and panel B (7 days). The doses of virus given per testis were: $4x10^8$ pfu (A and B), $1x10^7$ pfu (C and D), $1x10^6$ pfu (E and F), $1x10^5$ pfu (G and H). Arrow in panel B indicates a tubule that has been invaded by an unidentified population of cells. The arrows in panels C-H indicate sites of damage associated with infection with adenovirus. The untreated contralateral testis from the same animal as the testis in panel A is shown as a control in panel I. Images are 20x magnification and the scale bar represents 100 μm.

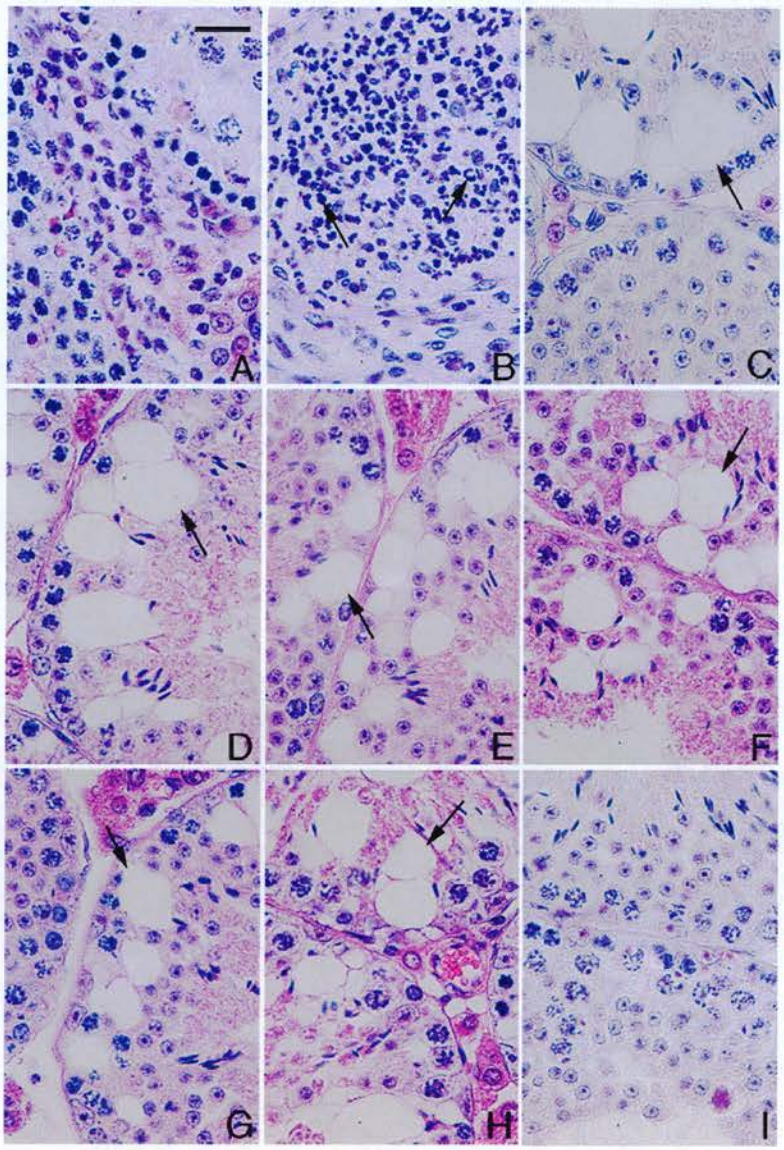


Figure 4-9: Haemoxylin and Eosin staining of adult mouse testes infected with a *GFP* adenoviral construct at a range of doses. Seminiferous tubules of the testes were specifically infected by intra-testicular injection. Testes were collected 4 days after infection except for those in panels A and I (3 days) and panel B (7 days). The doses of virus given per testis were: $4x10^8$ pfu (A and B), $1x10^7$ pfu (C and D), $1x10^6$ pfu (E and F), $1x10^5$ pfu (G and H). Arrows in panel B indicate potential immune cells, neutrophil (top arrow) and lymphocyte (bottom arrow), that have invaded the tubule. The arrows in panels C-H indicate sites of damage associated with infection with adenovirus. The untreated contralateral testis from the same animal as the testis in panel A is shown as a control in panel I. Images are 100x magnification and the scale bar represents 20 μm.

4.3.2.2.2 Stage specific germ cell loss

Following the observation that pachytene spermatocytes were lost from infected tubules, leaving vacuoles in the seminiferous epithelium, the stages of the spermatogenic cycle at which these losses occur were determined using H&E stained sections. A survey of tubules with GC loss and vacuoles was carried out on testes infected at $1x10^6$ and $1x10^7$ pfu per testis. Vacuoles and associated loss of GC were observed in tubules at stages II-VI and IX-XI, examples of each stage are shown in Figure 4-10 (panels A-I). The stages at which GC loss was most frequently observed were stages V and VI. Despite a substantial number of tubules at stages VII and VIII in the sections from infected testes, all appeared to have a full GC complement (panels M-O).

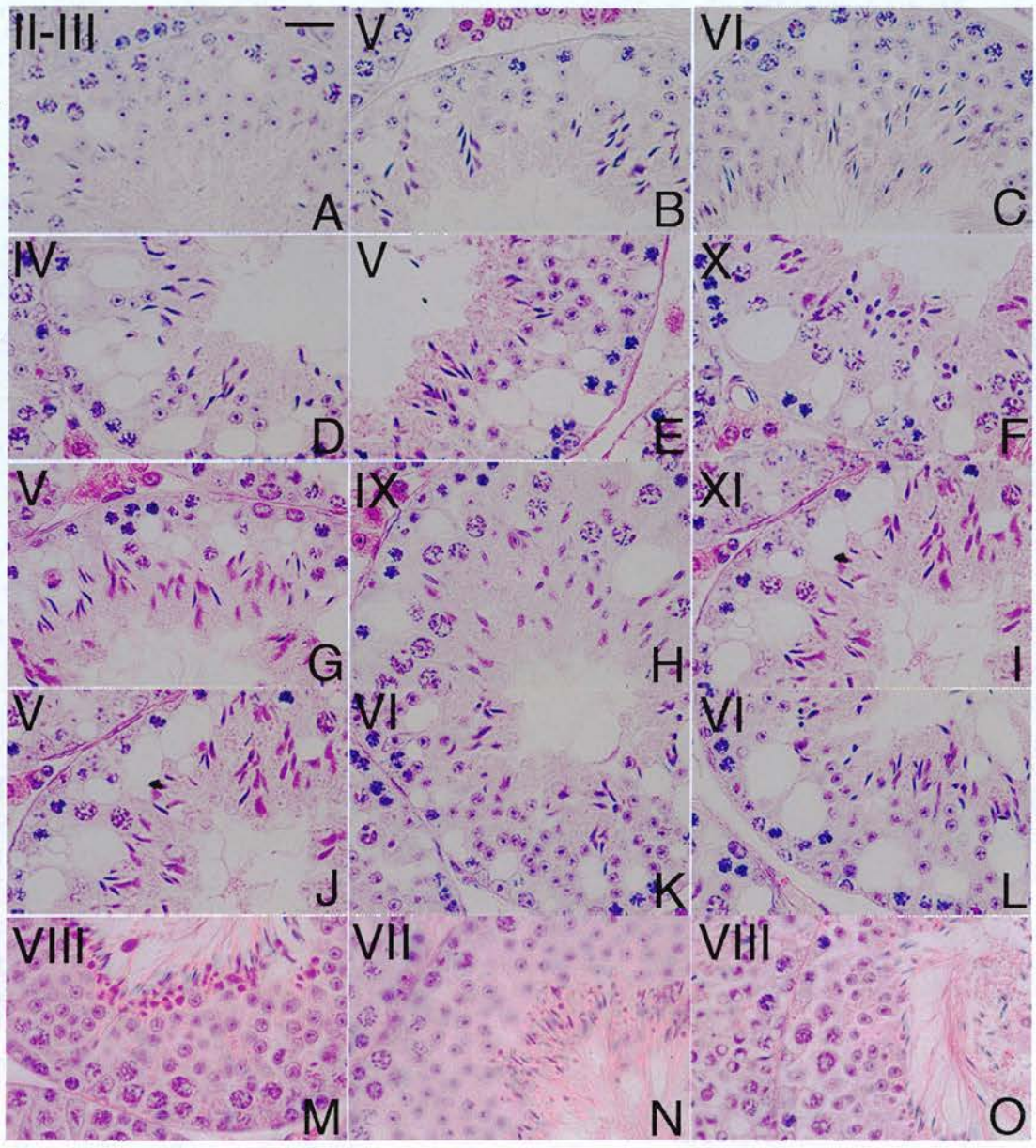


Figure 4-10: Stages at which damage is observed in haemoxylin and eosin stained adult mouse testes infected with a *GFP* adenoviral construct at $1x10^7$ (A-F, & M) and $1x10^6$ (G-L, & N-O) pfu per testis. The stage of spermatogenesis in each tubule is shown in the top left corner of every image. Images are 100x magnification and the scale bar represents 20 μm.

4.3.2.2.3 Sdmg-1

Sdmg-1 is expressed in the cytoplasm of SC and was detected in tissue sections by immunohistochemistry (Figure 4-11). Positive immunostaining for Sdmg-1 was seen in the seminiferous tubules of all the testes infected with adenoviral particles irrespective of the number of pfu injected. In those testes injected with $4x10^8$ pfu and recovered after 7 days (panel A) the number of identifiable stained cells was reduced, and in testes collected after 3 days (panel B) the amount of staining was similar to that of uninfected testes (panel I). In testes injected with $1x10^5$-$1x10^7$ pfu (panels C-H) immunopositive SC cytoplasm extended from the basement of the tubules to the lumen. Within these testes, the immunostaining was similar in damaged and undamaged tubules within the same section.

In tubules from uninfected testes the Sdmg-1 staining was highly organised with dark staining running from the basement membrane towards the lumen in narrow bands around the full circumference of the tubule (panel I). In testes infected with $4x10^8$ pfu the pattern was greatly disrupted. In testes collected 3 days after infection Sdmg-1 staining was seen throughout the tubule, including at the centre where the lumen had collapsed. Staining was disorganised consistent with disruption of testicular architecture (panel A). In the testes recovered 7 days after intra-testicular injection, what little immunopositive staining was detected was disorganised (panel B). In the tubules injected with $1x10^5$ - $1x10^7$ pfu (panels C-H) the pattern of Sdmg-1 expression was interrupted by the gaps in the seminiferous epithelium. Although Sdmg-1 was expressed in the same narrow 'banded pattern' running from the basement towards the lumen it followed the contours of the damage to the epithelium, in tubules lacking disruption to their epithelium the Sdmg-1 distribution was unaltered.

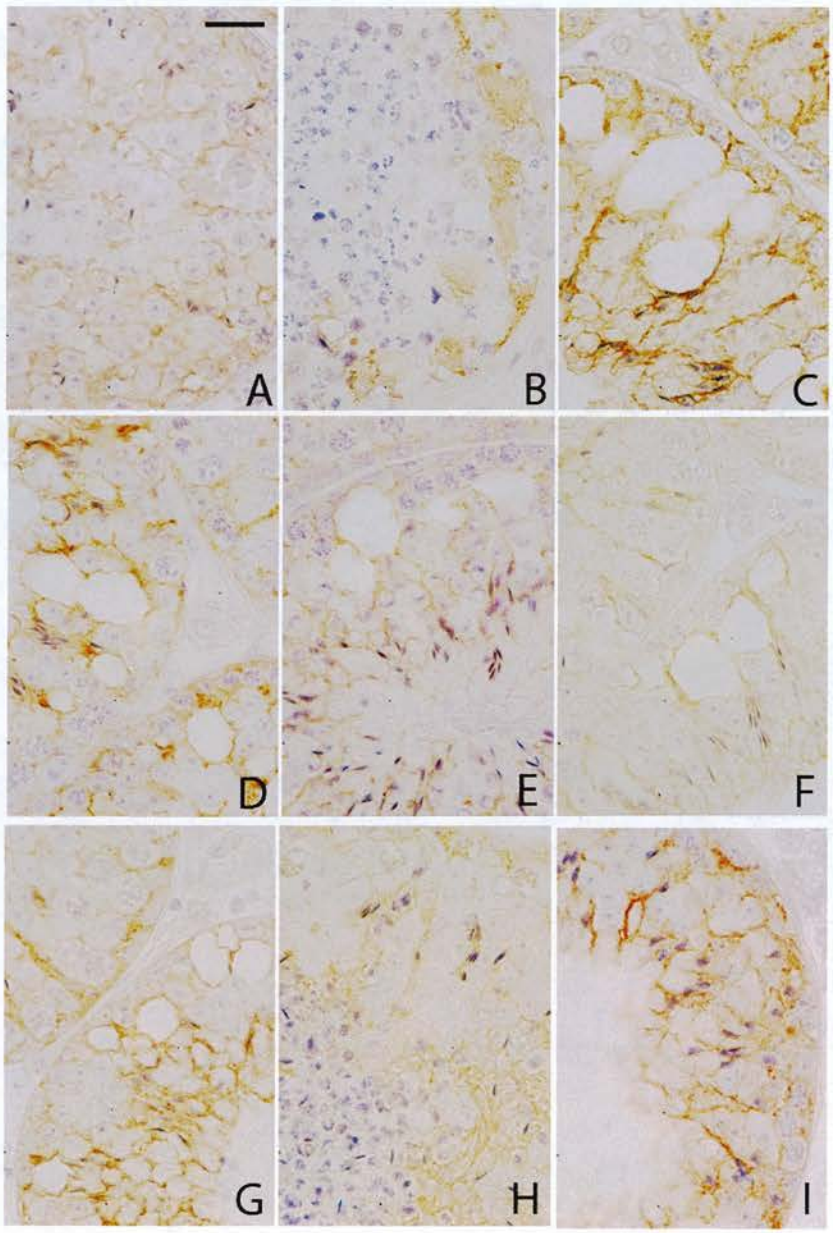


Figure 4-11: Immunological staining of Sdmg-1 (Sc-marker) expression in mouse testes infected with *GFP* adenoviral construct at a range of doses. Seminiferous tubules of the testes were specifically infected by intra-testicular injection. Testes were collected 4 days after infection except for those in panel A (3 days) and panel B (7 days). The doses of virus given per testis were: $4x10^8$ pfu (A and B), $1x10^7$ pfu (C and D), $1x10^6$ pfu (E and F), $1x10^5$ pfu (G and H). The untreated contralateral testis from the same animal as the testis in panel A is shown as a control in panel I. Images are 100x magnification and the scale bar represents 20 μm.

4.3.2.2.4 Junctional Complexes

4.3.2.2.4.1 Espin and connexin

Damage to seminiferous tubule structure and loss of GC, was associated with infection of testes by $1x10^5$ – $4x10^8$ pfu/testis. In order to determine whether this was also associated with disturbance in the physical/junctional interactions between SC and GC, sections were stained for espin (ectoplasmic specialisation) and Cx43 (Gap junction). The uninfected contra-lateral testes recovered from treated animals at the same time as the infected testes served as controls (Figure 4-12 and Figure 4-13 panels C, F, I and L). Expression of espin and Cx43 in infected and uninfected testes counterstained with DAPI at x40 and x80 magnification is shown in Figure 4-12 and Figure 4-13 respectively.

No immunoexpression of Cx43 in tubules of testes infected with $4x10^8$ pfu/testis was detected in testes recovered at 3 (panel A) and 7 days (panel B) after infection. Expression of espin was detected at 3 days but was absent from testes collected at 7 days. In testes injected with $1x10^5$ – $1x10^7$ pfu/testis and recovered 4 days later the expression of Cx43, but not of espin, was reduced in tubules with vacuoles in their epithelium (marked by * in panels). Expression of Cx43 in these tubules was either lost or diminished significantly, but the loss of Cx43 expression was only seen in tubules with vacuoles; the expression of Cx43 in adjacent undisrupted tubules was not affected. The loss of Cx43 expression was greatest in tubules infected with $1x\ 10^6$ (panels G and H) and $1x10^7$ (panels D and E) pfu/testis, in which only very limited expression of Cx43 was observed. In testes infected with $1x10^5$ pfu (panels J and K) expression of Cx43 was reduced compared to uninfected testes but some tubules which had some loss of GC showed moderate Cx43 expression.

Although expression of espin was still detected in infected testes (except in testes infected with $4x10^8$ pfu and recovered 7 days later) the distribution of espin immunostaining was altered by the presence of vacuoles in the epithelium with positive

staining apparent at the boundaries of the vacuoles (indicated by arrows). In the testes infected with $4x10^8$ pfu/testis where espin was detected at the peripheries of the tubules expression was maintained but appeared disorganised towards the centre of the tubule.

In control testes and those tubules with a normal GC complement in infected testes co-expression of espin and Cx43 was detected at the peripheries of the tubule (yellow fluorescence). Co-expression of espin and Cx43 was also observed in disrupted tubules of testes infected with $1x10^5$ pfu/testis, when expression of Cx43 was only slightly diminished.

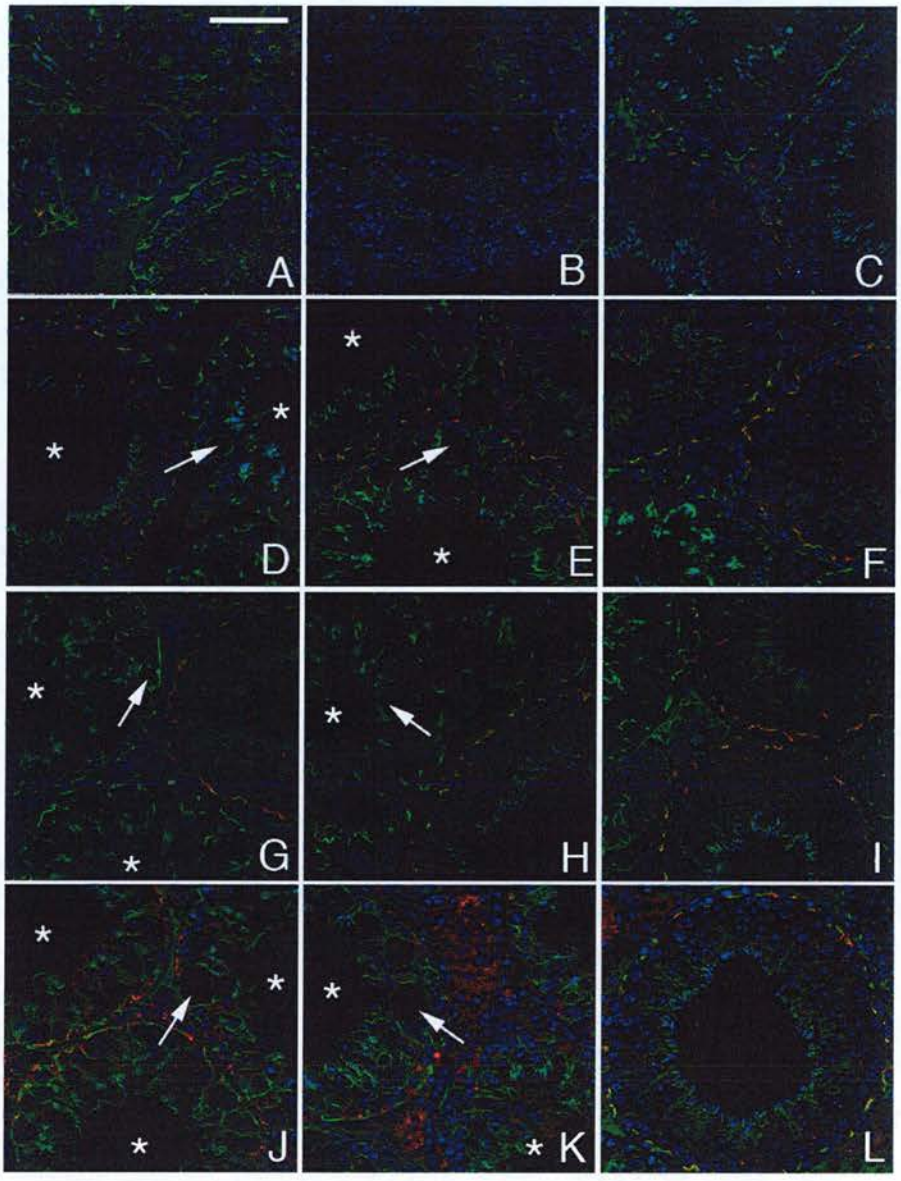


Figure 4-12: Fluorescent immunohistochemistry for espin (green) and Cx43 (red) in mouse testes infected with *GFP* adenoviral construct at a range of doses. Seminiferous tubules with disrupted epithelium are denoted by a *, arrows indicate the location of vacuoles. Testes were collected 4 days after infection except for those in panels A and C (3 days) and panel B (7 days). The doses of virus given per testis were: $4x10^8$ pfu (A and B), $1x10^7$ pfu (D and E), $1x10^6$ pfu (G and H), $1x10^5$ pfu (J and K). An untreated contralateral testis taken from a treated animal at each dose is shown in panels C, F, I and L. Each section was counterstained with DAPI nuclear marker (blue). Images are at x40 magnification and the scale bar represents 50 μm.

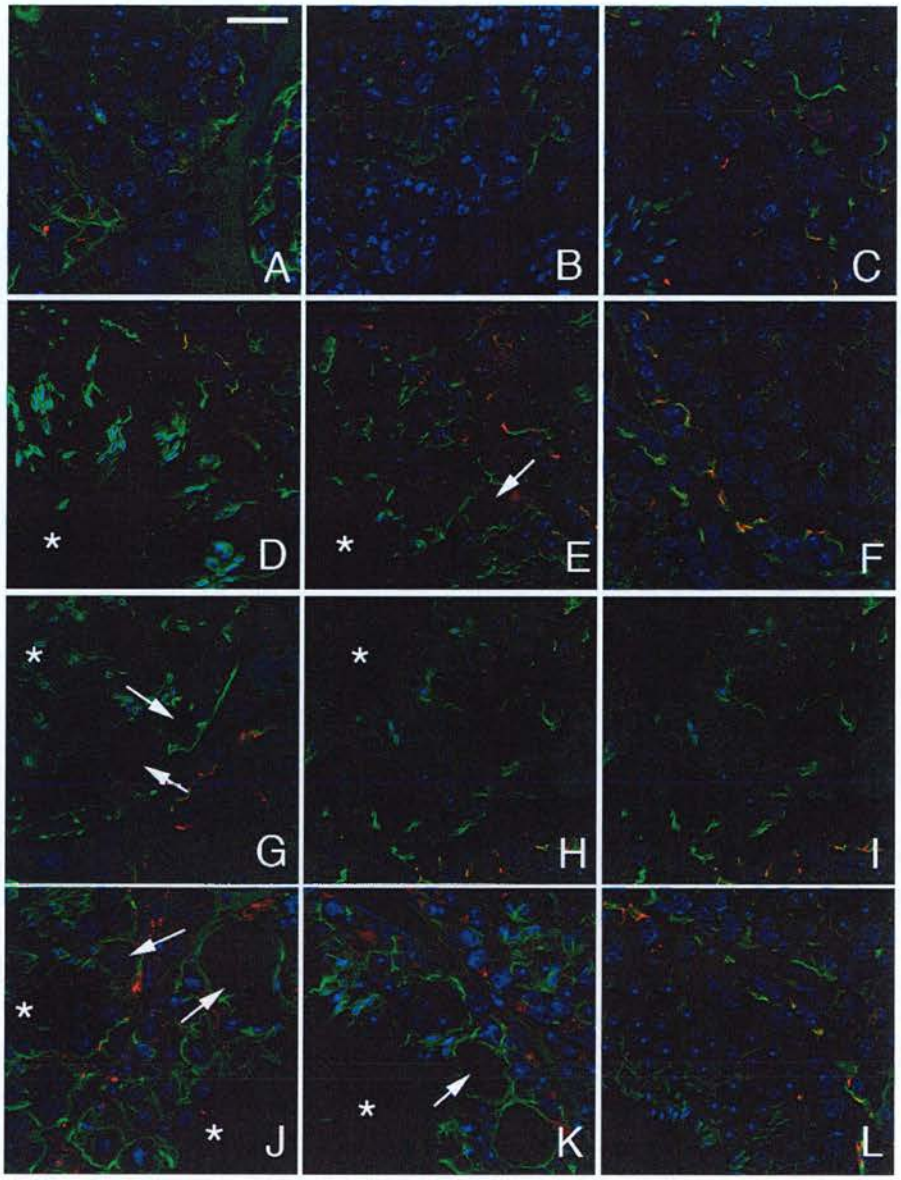


Figure 4-13: Fluorescent immunohistochemistry for espin (green) and Cx43 (red) in mouse testes infected with *GFP* adenoviral construct at a range of doses. Seminiferous tubules with disrupted epithelium are signified by a *, arrows indicate the location of vacuoles. Testes were collected 4 days after infection except for those in panels A and C (3 days) and panel B (7 days). The doses of virus given per testis were: $4x10^8$ pfu (A and B), $1x10^7$ pfu (D and E), $1x10^6$ pfu (G and H), $1x10^5$ pfu (J and K). An untreated contralateral testis taken from a treated animal at each dose is shown in panels C, F, I and L. Each section was counterstained with DAPI nuclear marker (blue). Images are at x80 magnification and the scale bar represents 20 μm.

4.3.2.3 Evidence of hypoxia and cell death

4.3.2.3.1 HIF-1α

Hypoxia induced factor 1α (Hif-1α) is a marker of hypoxia and its expression was visualised by immunohistochemistry (Figure 4-14). Hif-1α was immunolocalised to GCs of testes infected with $4x10^8$ pfu/testis and recovered 3 or 7 days after infection (panels A and B respectively), as well as testes recovered 4 days after infection with $1x10^5$ pfu (panels G and H). Testes infected for 4 days with $1x10^5$ pfu/testis had more intense immunostaining than those infected with $4x10^8$ pfu. Testes infected with $1x10^6$ and $1x10^7$ pfu and recovered 4 days later did not contain Hif-1α positive staining within the infected tubules (panels C-F) and neither did uninfected testes recovered 3 days after injection of the contra-lateral testis (panel I).

Immunopositive Hif-1α staining was observed in some LC within the interstitium of tubules injected at the four viral doses/testis, and was also expected in LC of uninfected control testes due to reports in previous literature.

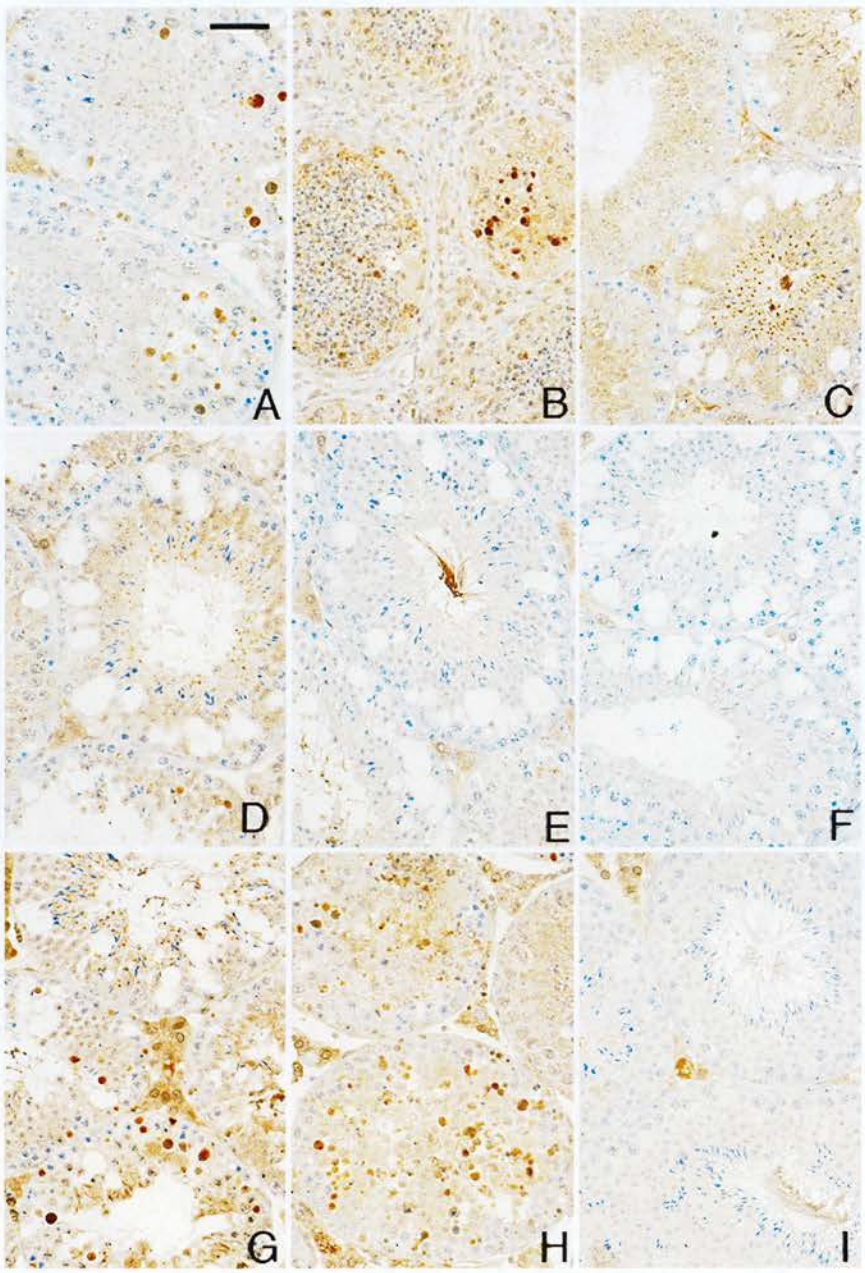


Figure 4-14: Immunohistochemical localisation of HIF-1α in mouse testes infected with *GFP* adenoviral construct at a range of doses. Seminiferous tubules of the testes were specifically infected by intra-testicular injection. Testes were collected 4 days after infection except for those in panel A (3 days) and panel B (7 days). The doses of virus given per testis were: $4x10^8$ pfu (A and B), $1x10^7$ pfu (C and D), $1x10^6$ pfu (E and F), $1x10^5$ pfu (G and H). The untreated contralateral testis from the same animal as the testis in panel A is shown as a control in panel I. Images are 40x magnification and the scale bar represents 50 μm.

4.3.2.3.2 Cleaved-Caspase-3

Immunohistochemistry for cleaved-caspase 3, a marker of apoptotic cell death, was performed on sections from mouse testes infected with $1x10^5$ – $4x10^8$ pfu/testis (Figure 4-15). Weak immunopositive staining for cleaved-caspase 3 was only found within the seminiferous tubules in sections of testes injected with $4x10^8$ pfu and recovered 3 or 7 days after infection (panels A and B respectively) or injected with $1x10^5$ pfu (panels G and H). The location and number of stained cells within the seminiferous tubules indicated that they were GC. The number of cleaved-caspase 3 positive cells present in testes injected with $4x10^8$ pfu was greater in the testes recovered 7 days after injection compared to those collected after 3 days. No positive staining was found in testes infected with $1x10^6$ - $1x10^7$ pfu/testis (panels C-F), or in the uninfected contra-lateral testis associated with the testis infected with $4x10^8$ pfu and collected 7 days after infection (panel I). The absence of any cleaved-caspase in these testes was unexpected because a low incidence of cell death, marked by cleaved-caspase, would be expected even within a healthy population of testicular cells.

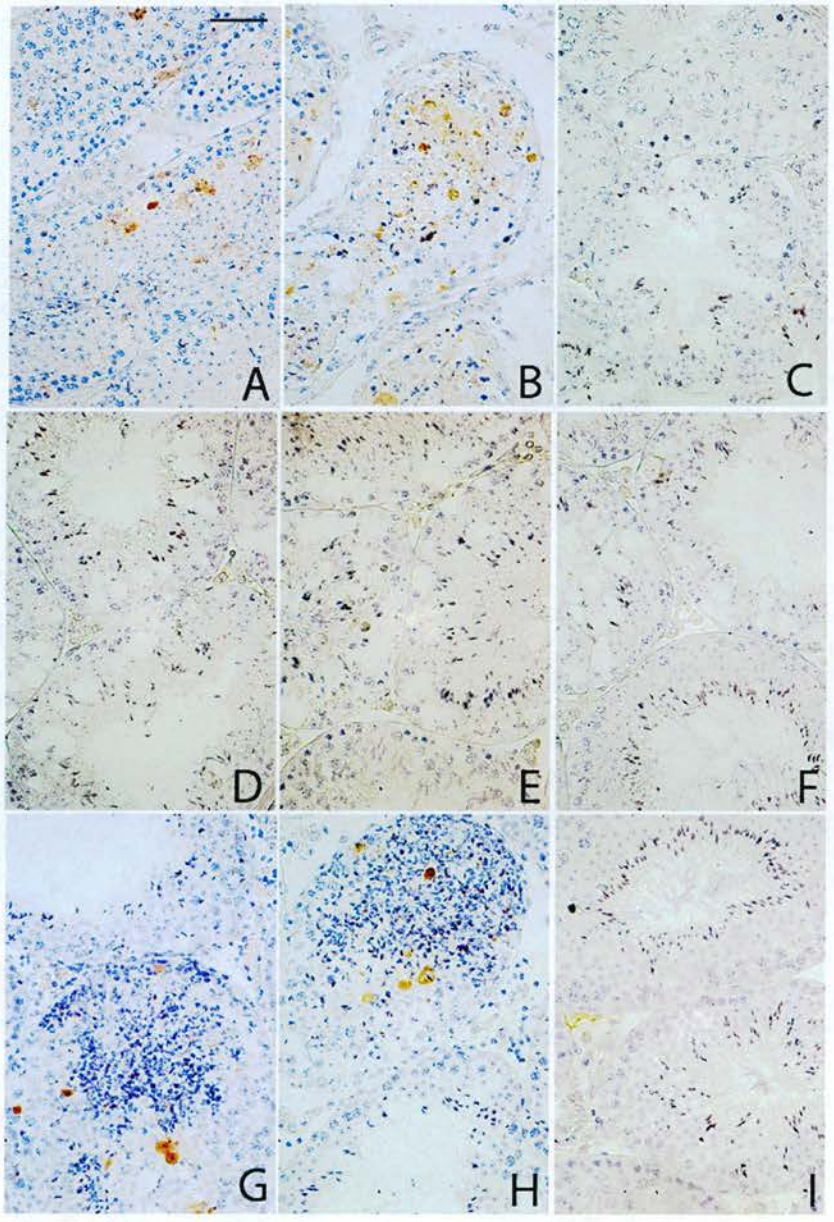


Figure 4-15: Immunological staining of cleaved-caspase 3 expression in mouse testes infected with a *GFP* adenoviral construct at a range of doses. Seminiferous tubules of the testes were specifically infected by intra-testicular injection. Testes were collected 4 days after infection except for those in panel A (3 days) and panel B (7 days). The doses of virus given per testis were: $4x10^8$ pfu (A and B), $1x10^7$ pfu (C and D), $1x10^6$ pfu (E and F), $1x10^5$ pfu (G and H). The untreated contralateral testis from the same animal as the testis in panel A is shown as a control in panel I. Images are 40x magnification and the scale bar represents 50 μm.

4.3.2.3.3 Apoptag

Apoptag staining, also known as the Tunel assay, detects single-stranded DNA and therefore the presence of DNA stand breaks associated with cells undergoing apoptosis (Figure 4-16). Only mouse testes infected with adenovirus at $4x10^8$ pfu/testis and collected 3 days or 7 days after infection contained Apotag positive cells (panels A and B, Figure 4-16). The immunopositive cells were localised within the seminiferous tubules and were assumed to be GC or immune cells. The uninfected contra-lateral testis associated with the testis recovered after 3 days showed no positive cells (panel I). Isolated cells with faint staining were found in sections from testes collected 4 days after infection with $1x10^5$ – $1x10^7$ pfu/testis, the positive cells are indicated by arrows in panels C – H in Figure 4-16. The proportion of Tunel-positive cells in these section was very low and no greater than would be expected in an uninfected healthy testis, only the proportion of cells seen in testes infected with $4x10^8$ pfu was significant.

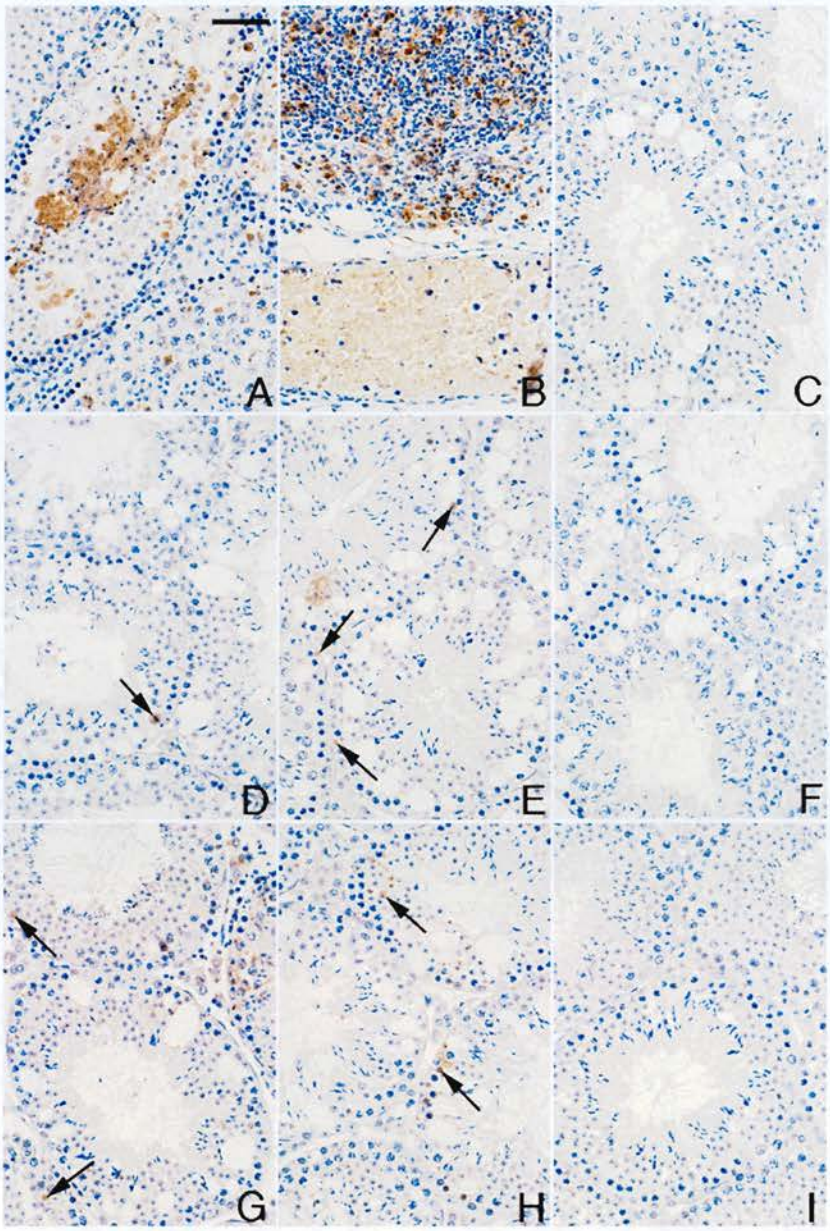


Figure 4-16: Immunological staining of DNA-strand breaks (Tunel assay) in mouse testes infected with a *GFP* adenoviral construct at a range of doses. Seminiferous tubules of the testes were specifically infected by intra-testicular injection. Testes were collected 4 days after infection except for those in panel A (3 days) and panel B (7 days). The doses of virus given per testis were: $4x10^8$ pfu (A and B), $1x10^7$ pfu (C and D), $1x10^6$ pfu (panels E and F), $1x10^5$ pfu (G and H). The untreated contralateral testis from the same animal as the testis in panel A is shown as a control in panel I. Isolated positively stained cells are indicated by arrows. Images are 40x magnification and the scale bar represents 50 μm.

4.3.2.4 Evidence of immune cell invasion

4.3.2.4.1 MPO

Testis sections were stained with an antibody to myeloperoxidase (MPO) to detect neutrophils in testes infected with $1x10^5$ – $4x10^8$ pfu (Figure 4-17 and Figure 4-18). MPO-positive cells were observed in testes infected with $4x10^8$ and $1x10^5$ pfu (panels A, B, G and H), and were absent from non-infected testes (panel I) and those infected with $1x10^6$ and $1x10^7$ pfu (panels C – F). There was more invasion by MPO-positive cells in testes infected with $4x10^8$ pfu than those infected with $1x10^5$, but MPO positive cells were found both within the interstitium and the tubules at both doses. The testes infected with $4x10^8$ pfu and recovered 7-days after infection (panel B) were associated with a greater degree of disruption and with greater invasion by the MPO-positive cells than testes collected only 3 days after the same dose of virus (panel A).

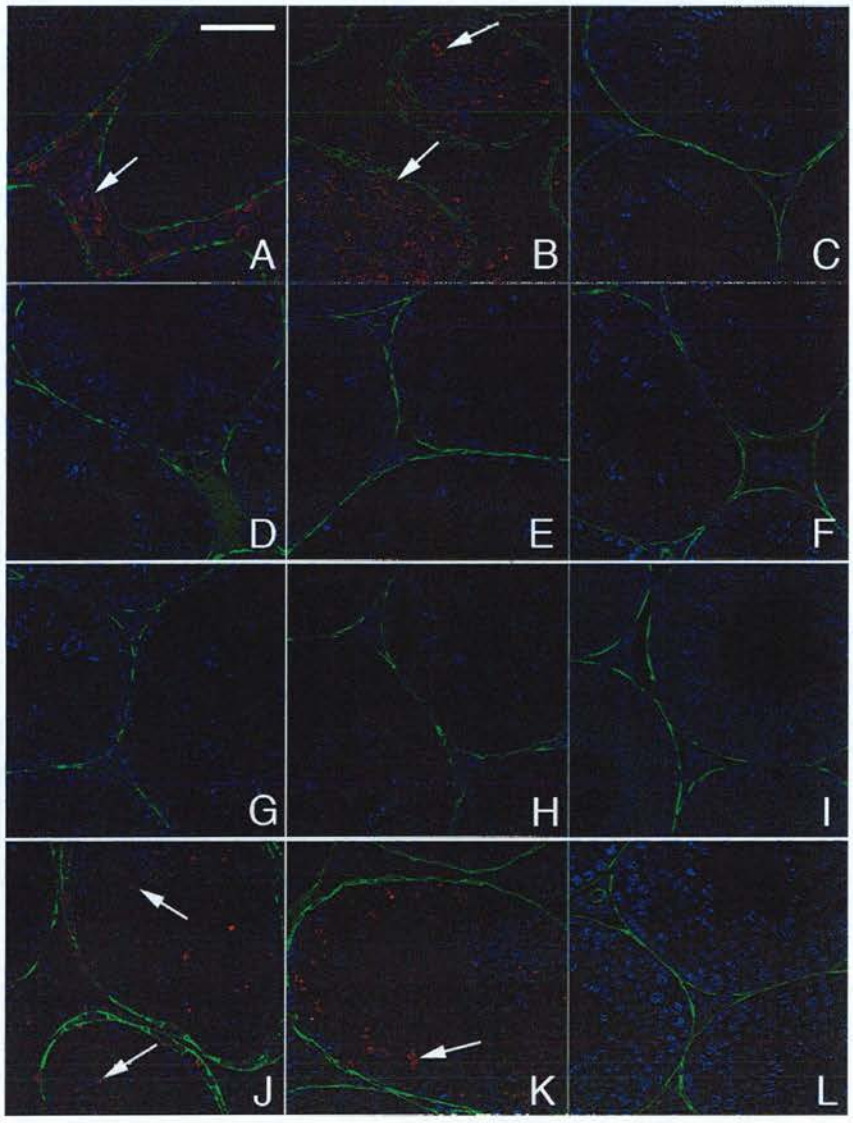


Figure 4-17: Fluorescent immunohistochemistry for myeloperoxidase (MPO) neutrophil marker (red) and smooth muscle actin (green) in mouse testes infected with a *GFP* adenoviral construct at a range of doses. Testes were collected 4 days after infection except for those in panels A and C (3 days) and panel B (7 days). The doses of virus given per testis were: $4x10^8$ pfu (A and B), $1x10^7$ pfu (D and E), $1x10^6$ pfu (G and H), $1x10^5$ pfu (panels J and K). The arrow in panel A indicates MPO-positive cells in the testes' interstitium, those in panels B, J and K denote MPO-positive cells within the tubule itself. An untreated contralateral testis taken from a treated animal at each dose is shown in panels C, F, I and L. Each section was counterstained with DAPI nuclear marker (blue). Images are at x40 magnification and the scale bar represents 50 μm.

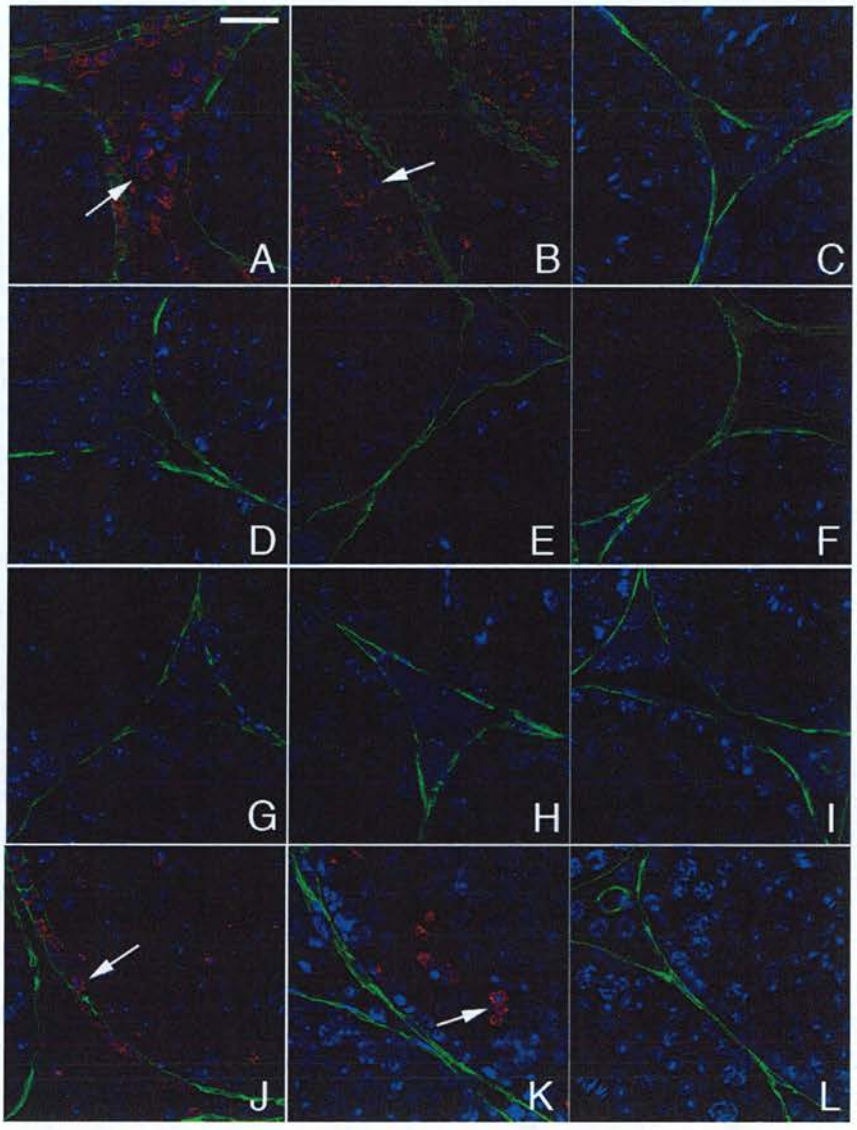


Figure 4-18: Fluorescent immunohistochemistry for myeloperoxidase (MPO) neutrophil marker (red) and smooth muscle actin (green) in mouse testes infected with a *GFP* adenoviral construct at a range of doses. Testes were collected 4 days after infection except for those in panels A and C (3 days) and panel B (7 days). The doses of virus given per testis were: $4x10^8$ pfu (A and B), $1x10^7$ pfu (D and E), $1x10^6$ pfu (G and H), $1x10^5$ pfu (J and K). The arrow in panel A indicates MPO-positive cells in the testes' interstitium, those in panels B, J and K denote MPO-positive cells within the tubule itself. An untreated contralateral testis taken from a treated animal at each dose is shown in panels C, F, I and L. Each section was counterstained with DAPI nuclear marker (blue). Images are at x80 magnification and the scale bar represents 20 μm.

4.3.2.4.2 CD68

Macrophages in tissue sections from mouse testes infected with $1x10^5$ – $4x10^8$ pfu/testis were detected by fluorescent immunohistochemistry for CD68 (Figure 4-19 and Figure 4-20). A small number of CD68-positive cells were observed in the interstitium of both infected and uninfected testes, however testes infected with $1x10^5$ or $4x10^8$ pfu contained increased numbers of CD68-positive cells. Testes infected with $1x10^5$ pfu/testis had a greater number of CD68-positive cells present but they were restricted to the interstitium, in testes injected with $4x10^8$ pfu/testis CD68-positive cells also infiltrated the seminiferous tubules in addition to increased numbers of cell in the interstitium. Co-staining with SMA (PTM cell marker) at the base of the seminiferous tubules revealed disruption to tubular architecture associated with increased numbers of CD68-positive cells in testes collected 3 days after injection with $4x10^8$ pfu. In contrast to infiltration by MPO-positive cells (section 4.3.2.4.1), testes infected with $4x10^8$ pfu appeared to contain a greater number of CD68-positive cells in their interstitium and seminiferous tubules when testes were collected 3 days rather than 7 days after infection.

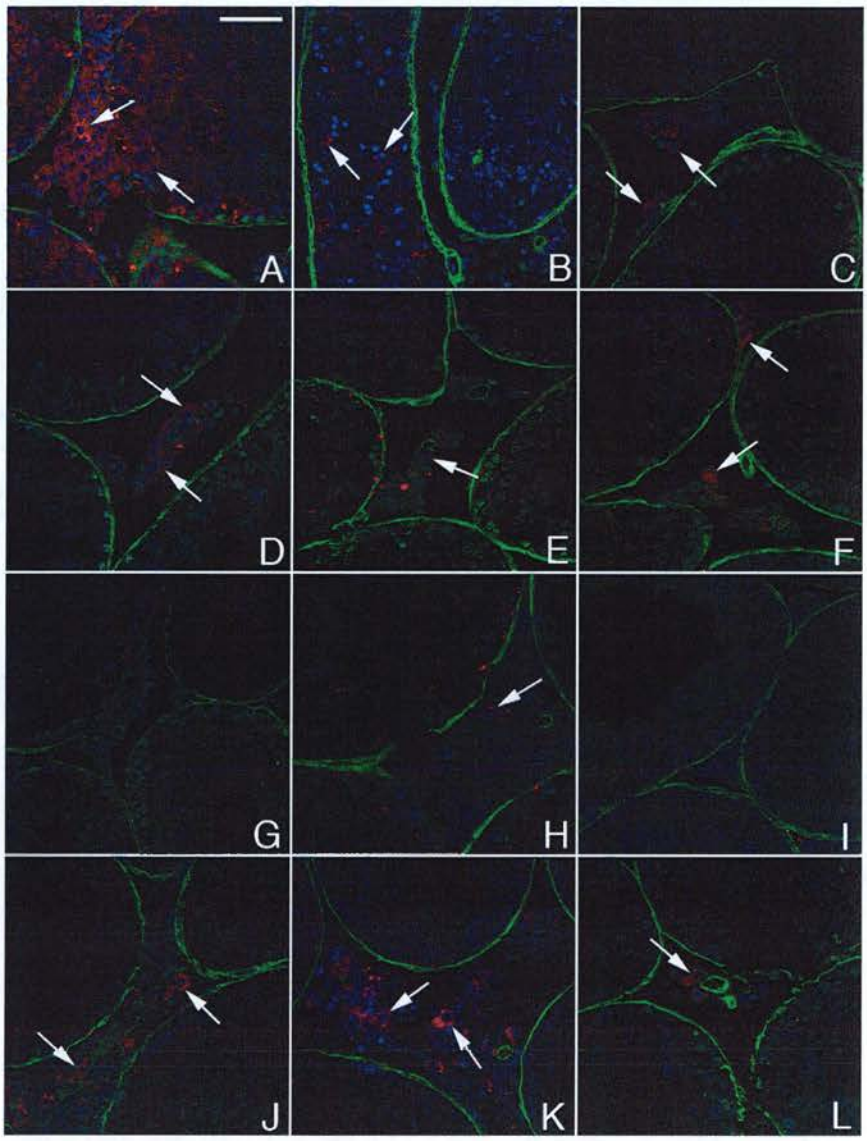


Figure 4-19: Fluorescent immunohistochemistry for CD68 macrophage marker (red) and smooth muscle actin (green) in mouse testes infected with a *GFP* adenoviral construct at a range of doses. Testes were collected 4 days after infection except for those in panels A and C (3 days) and panel B (7 days). The doses of virus given per testis were: $4x10^8$ pfu (A and B), $1x10^7$ pfu (D and E), $1x10^6$ pfu (G and H), $1x10^5$ pfu (J and K). Arrows indicate CD68-positive cells, in the interstitium and seminiferous tubules of infected testes and in the interstitium of untreated control testes. An untreated contralateral testis taken from a treated animal at each dose is shown in panels C, F, I and L. Each section was counterstained with DAPI nuclear marker (blue). Images are at x40 magnification and the scale bar represents 50 μm.

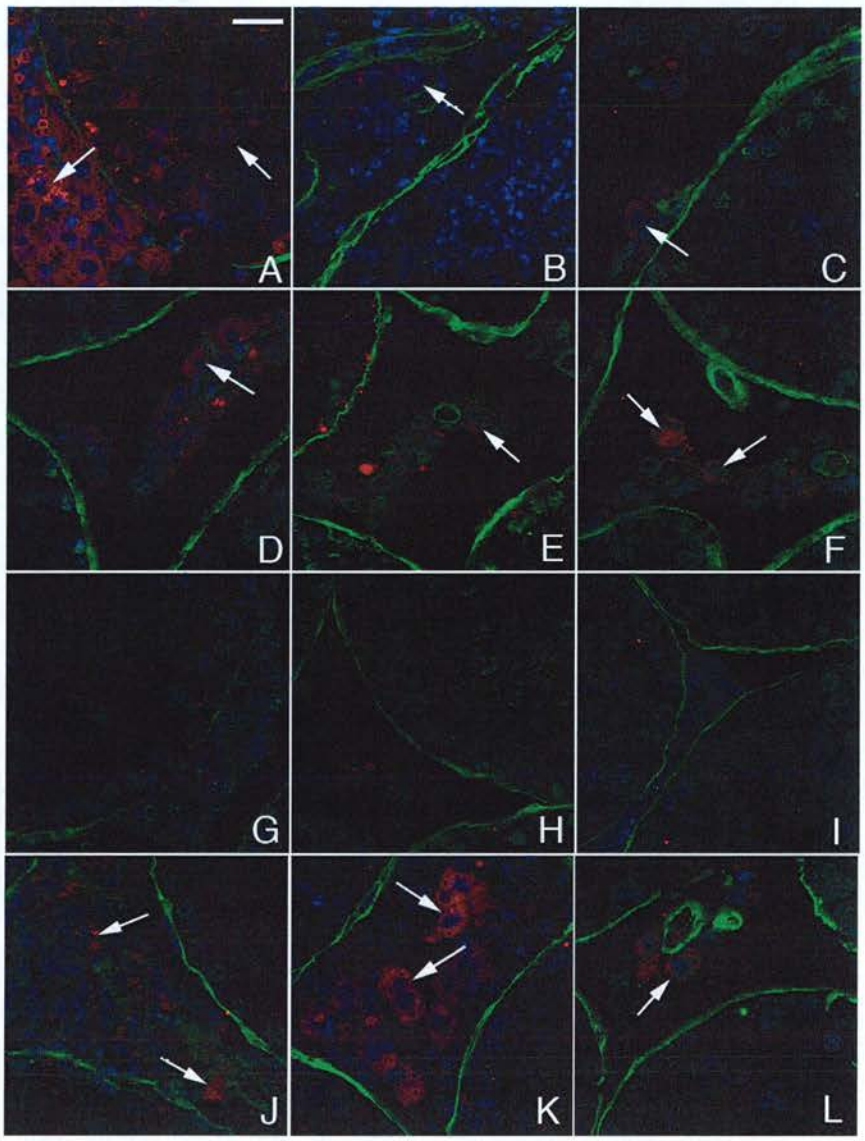


Figure 4-20: Fluorescent immunohistochemistry for CD68 macrophage marker (red) and smooth muscle actin (green) in mouse testes infected with a *GFP* adenoviral construct at a range of doses. Testes were collected 4 days after infection except for those in panels A and C (3 days) and panel B (7 days). The doses of virus given per testis were: $4x10^8$ pfu (A and B), $1x10^7$ pfu (D and E), $1x10^6$ pfu (G and H), $1x10^5$ pfu (J and K). Arrows indicate CD68-positive cells found in the interstitium and seminiferous tubules of infected testes and only the interstitium of control testes. An untreated contralateral testis taken from a treated animal at each dose is shown in panels C, F, I and L. Each section was counterstained with DAPI nuclear marker (blue). Images are at x80 magnification and the scale bar represents 20 μm.

4.4 Discussion

In the course of investigating the feasibility of adenoviral constructs for introduction of constructs specifically into SC *in vivo* it was found that *in vitro* SC, SK11 cells, efficiently expressed reporter constructs introduced via adenoviral infection. The moderate quantities of plaque-forming viral particles required for substantial expression of *LacZ* or *GFP* reporter genes were not sufficient to cause cell death *in vitro*, however infection with higher concentrations of virus did have a cytotoxic effect. *In vivo* use of the same adenoviral GFP-construct resulted in SC-specific expression when the virus was introduced by intra-testicular injection and no expression occurred in the GC. *In vivo* infections resulted in highly variable expression of the reporter protein between adjacent tubules. Infection at MOIs which had no impact on SC function *in vitro* were associated with low levels of expression of GFP and were associated with detrimental effects including germ cell loss, structural disruption and infiltration by macrophages and neutrophils.

4.4.1 *In Vitro*

The present study demonstrates that an SC cell line maintained *in vitro* (SK11) can readily be infected by adenoviral vectors and will efficiently express transgenes carried by these constructs, a finding that has been reported previously in primary SC cultures (Blanchard and Boekelheide, 1997; Scobey et al., 2001) and in the immortalised Tm4 SC line (Fleming et al., 2003b). Although we were able to show that the majority of cells would express the transgene at MOIs of 50-75, the MOIs required for significant transgene expression were many fold higher in the study by Blanchard and Boekelheide (Blanchard and Boekelheide, 1997). For example they claimed that they did not observe any morphological signs of cell disruption or cell detachment from the culture surface using infections at 5000 MOI, whereas we found that infection of SK11 cells at only 85 MOI caused cell detachment. Fleming et al, who also used immortalised cells found 10 and 100 MOI resulted in efficient expression of the transgene (Fleming et al., 2003b). These differences may reflect differences in the SC and viral constructs used as the SK11 and Tm4 cells are immortalised, whilst Blanchard et al used purified primary

SC cultures and immortalisation of the SK11 cells may have enhanced their sensitivity to viral infection. There may also have been differences between the adenoviral constructs in each study, their method of purification, and indeed their purity.

Differentiated SK11 cells, cultured at 39°C, secreted more IL-6 protein than did proliferative cells cultured at 34°C. This result is in agreement with previous studies in which IL-6 expression was greater in primary cultures of SC derived from pubertal rats, compared to those from pre-pubertal animals (Syed et al., 1992). The SC of the pubertal rats should be mature and are comparable to the differentiated SK11 cells, whilst the proliferative SK11 cells should resemble the pre-pubertal cells. A similar pattern of low pre-pubertal and higher pubertal expression has also been reported for IL-1α, which as a key factor in the regulation of IL-6, and could be investigated to complement the increased IL-6 expression found in differentiated SK11 cells (Gérard et al., 1991).

Although the pattern of release of IL-6 by the SK11 cells paralleled expression in SC in the immature and mature states, the SK11 cells did not respond to infection by adenoviral constructs by raising expression of IL-6. This is in contrast to primary cultures of SC which show an increase in expression of IL-6 in response to treatment with LPS or residual bodies (Riccioli et al., 1995; Syed et al., 1993). Although this experiment does not provide a direct comparison to adenoviral infection, it does show that primary SC cultures maintain a capacity to respond to inflammatory stimulation that SK11 cells appear to lack. In primary cells the increased expression of IL-6 expression is in response to increased autocrine IL-1α signalling (Syed et al., 1995). Further studies are therefore required to determine whether viral infection of SK11 cells has any impact on IL-1α. The time course used in this study covered 48 hours and included time points at 6 and 24 hours after viral infection, it was at these times that LPS and residual bodies, respectively, induced maximal IL-6 expression in primary SC cultures (Syed et al., 1993), so it is unlikely that a virally induced IL-6 surge was missed.

4.4.2 *In Vivo*

Detection of the GFP reporter protein in SC, but not in GC, within the tubules of testes injected with adenoviral constructs via the efferent ductules demonstrates the specificity of adenoviral infection for SC, and the capacity of SC to express transgenes introduced via adenoviral vectors. These findings were in agreement with the *in vitro* data presented above and *in vitro* and *in vivo* data presented in previous studies on rats and mice (Blanchard and Boekelheide, 1997; Fleming et al., 2003a; Fleming et al., 2003b; Ikawa et al., 2002; Kanatsu-Shinohara et al., 2002; Scobey et al., 2001). Blanchard and Boekelheide (Blanchard and Boekelheide, 1997) used *in vitro* co-cultures of SC and GC and showed that at viral concentrations sufficient for efficient expression in SC, only a very small minority (<1%) of GC showed expression of an adenoviral transgene. In previous studies adenoviral constructs were introduced into seminiferous tubules *in vivo* by similar methods to those used in the current study and included injection into the rete testis, seminiferous tubule lumen or efferent ductile. In these studies SC-specific expression was achieved (Blanchard and Boekelheide, 1997; Kanatsu-Shinohara et al., 2002; Scobey et al., 2001), and the GC of the infected tubules did not express the viral transgene (Scobey et al., 2001). In addition, systemic infection of mice with an adenovirus bearing a GC-specific promoter and reporter protein construct resulted in no germ line expression (Peters et al., 2001).

The efficiency of *in vivo* transgene expression in this study was not as high as *in vitro*, as only a small proportion of tubules contained SC expressing the *GFP* transgene, and not all SC within each immunopostitive tubule expressed GFP, especially at the lower viral concentrations. Incomplete coverage of transgene expression was also reported in previous studies with between 5 and 34 percent of tubules expressing adenoviral transgenes (Blanchard and Boekelheide, 1997; Scobey et al., 2001). The reduced efficiency of *in vivo* expression at $1x10^5$ – $1x10^7$ pfu/testis, compared to *in vitro*, is likely to be a consequence of the reduced MOI (pfu per cell to be infected) achieved *in vivo* that are predicted to be between 0.05 and 5. These MOI assume the entire length of the seminiferous tubules was bathed by the injected viral solution that is unlikely. Even

if the virus reached only a fraction of the total length increasing the MOI several fold it would still be below 50, which was required *in vitro* for substantial transgene expression. Fleming et al suggest that introducing the virus via the rete testis results in high exposure at sites near the rete but that the viral titre is decreased at more distal sites due to dilution and uptake of the virus by proximal cells. In addition, the decline in viral concentration they describe would directly impact expression of the transgene because expression level is correlated with the number of copies of the transgene that enter each cell (Fleming et al., 2003b). The original intention was to use vectors expressing both target-specific miRNAs and GFP so that changes in cell expression/germ cell survival could be linked to sites of specific knockdown so patchy expression would not necessarily have been a drawback as it would have allowed direct comparison between infected and non-infected cells.

Expression of the transgene in infected cells was detected as early as 72 hours after infection, which was the earliest time point at which *GFP* transgene expression was investigated in this study, in previous studies expression was detected as early as 48 hours after infection and could still be maintained after 3 months (Blanchard and Boekelheide, 1997; Kanatsu-Shinohara et al., 2002). Adenoviral infection has not only been used for expression of reporters to demonstrate the cell-specificity of the technique as constructs expressing i) a *cyclic adenosine 3',5'-monophosphate response element-binding protein* (*CREB*) mutant and ii) *steel factor* (*kit ligand*), have been used in intra-testicular infections to either disrupt spermatogenesis in wild-type rats or to rescue spermatogenesis in infertile mice respectively (Kanatsu-Shinohara et al., 2002; Scobey et al., 2001).

In the present study dramatic responses to adenoviral infection were observed. The responses were apparent in testes 3, 4 and 7 days after infection. The testes can be separated into two groups based upon their responses to infection. The first group contains testes infected at the greatest dose ($4x10^8$ pfu/testis) in which the internal structure of the seminiferous tubule disintegrated, there was evidence of hypoxia and

apoptosis and invasion of the interstitium and sometimes the tubules by macrophages and neutrophils. The testes of the second group were infected at lower doses ($1x10^5$-$1x10^7$ pfu/testis) and exhibited less severe effects that were similar at the 3 doses. Instead of collapsing, the tubules maintained a distinct lumen and a distinguishable seminiferous epithelium but with apparent GC loss and co-incident 'vacuole' formation at the sites of cell loss. Infrequently, neutrophil and macrophage invasion was associated with infected testes at these lower doses. The types of disturbances described above were not reported in studies carried out prior to the start of the work presented here. In previous studies low levels of immune response were reported when adenoviral constructs were injected into seminiferous tubules, even when infection of the interstitium was avoided. Blanchard and Boekelheide (1997) reported an inflammatory response that began 10 days after adenoviral infection via the rete testis, and lasted around 8 days. The inflammatory event was marked by lymphocyte and plasma cell infiltration into the interstitium, and chromatin fragmentation in cells of the seminiferous epithelium. The immune response following infection within tubules had a shorter duration than that associated with injections into the interstitium, which lasted up to 30 days after infection but also included invasion by lymphocytes and plasma cells (Blanchard and Boekelheide, 1997). Injection of adenovirus via the rete testis was reported to result in limited necrosis and invasion by immune cells, which is in contrast to injection directly into the lumen of seminiferous tubules that was apparently associated with no tissue damage or immune cell invasion (Scobey et al., 2001). Injection into the rete testis was sometimes associated with leakage of virus into the interstitium (Blanchard and Boekelheide, 1997), which would explain the immunological response associated with testicular injection by this method. A study comparing the efficiency of numerous viral vectors for introduction of transgenes into SC reported a significant reduction in testis weight compared to control testes when adenovirus was used as the vector (Ikawa et al., 2002), but this result is in contrast to the majority of the existing literature.

The presence or absence of vacuoles in tubules at specific stages of the spermatogenic cycle suggested that tubules vary in susceptibility to infection dependent upon their germ cell complement. Differential susceptibility of SC to adenoviral infection has already been reported in rat testes, with SC in tubules at stage II-VI predicted to be the primary targets (Blanchard and Boekelheide, 1997). However in a later study from the same group, 80% of infection was suggested to occur in stage V-XII tubules (Fleming et al., 2003b). In the present study the testes were examined 4 days after infection, and it was shown that the only stages of tubules that were identified as lacking vacuoles were those in stages VII and VIII. Four days previously when the testes were initially infected these tubules would have been in stages I-III. The worst affected stages 4 days after infection were stages V and VI, which would have been stages XI and XII of the previous cycle at the time of infection. The position of the gaps in the seminiferous epithelium and the GC complement in affected tubules indicated that the GC lost following infection are pachytene spermatocytes. The loss of pachytene spermatocyte following viral infection suggests that development of zygotene spermatocytes was compromised following viral infection of the SC. Although the previous studies of Blanchard, Scobey and Kanatsu-Shinohara (Blanchard and Boekelheide, 1997; Kanatsu-Shinohara et al., 2002; Scobey et al., 2001) reported immunological responses to adenoviral infection, none of them showed any evidence of germ cell loss or the appearance of vacuoles in the seminiferous epithelium when constructs expressing reporter proteins were used. The sensitivity of zygotene spermatoctyes to disruption during inflammatory events has been reported previously in a study using a systemic injection of LPS and this study also reported significant losses of pachytene spermatocytes 7 days after treatment (Liew et al., 2007). The zygotene spermatocytes in the LPS study did not undergo apoptosis or premature release, instead their development appears to be delayed, and the study links the disruption in development of the zygotene spermatocytes to their loss when they reached the pachytene spermatocyte stage. In contrast to the studies presented here, LPS-treatment also resulted in loss of round spermatids, which were released prematurely into the tubule lumen together with the spermatocytes (Liew et al., 2007).

Evidence of apoptosis and hypoxia were detected in testes infected with $4x10^8$ pfu/testis, associated with immunopositive staining for activated caspase-3, DNA-strand breakage and Hif-1α. The cells exhibiting positive staining were GC, which are not infected directly by the adenoviral constructs suggesting that the effect was secondary to the impact of viral infection on SC function. The expression of markers for hypoxia and apoptosis in these testes could be a consequence of the disrupted structure of their tubules. The distribution of the SC cytoplasm is dramatically altered in these tubules, as visualised in Figure 4-11, resulting in reduced and often total loss of contact between GC and their supporting SC. The compact structure of the disrupted tubules which lacked a distinct lumen following viral infection could reduce the oxygen permeability of the tubule as a whole and combined with loss of GC-SC contact could result in a reduction in oxygen concentration, i.e. hypoxia, within the tubule and thereby compromise the GC. Hypoxia results in stabilisation and build-up of the Hif-1α protein that may then bind hypoxic response elements in genes some of which are components of the apoptotic pathway (Carmeliet et al., 1998). In light of the association between apoptosis and hypoxia the presence of signs of apoptosis (activated caspase-3 and DNA-strand breaks) in GC that also express hypoxia markers is logical. In the absence of contact between GC and SC, the GC could also be deprived of essential factors supplied by SC that could contribute to induction of apoptosis in these cells. The presence of fragmented chromatin, indicating apoptosis, following adenoviral infection was previously reported in cells of the seminiferous epithelium, but within these tubules no evidence of apoptosis was reported in GC (Blanchard and Boekelheide, 1997), other studies using the Tunel assay revealed only normal levels of GC apoptosis following adenoviral infection (Scobey et al., 2001). However apoptosis of GC has been reported in inflamed rat testes induced by infection with LPS (Liew et al., 2007). Germ cell death also occurs in testes exhibiting experimental autoimmune orchitis (EAO) and includes the activation of caspase-3 in these cells (Theas et al., 2003). Further relevance in relation to findings of the current study are limited because although both are examples of orchitis the EAO is induced by priming the host immune system using host antigens

including those of haploid and meiotic GC meaning that the animal is specifically sensitised to these cell types. In addition, autoimmune orchitis requires up to fifty days after stimulation to induce apoptosis (Theas et al., 2003), whereas apoptosis was observed within 72 hours in this study.

Hif-1α expression was detected in tubules of testes injected with $1x10^5$ pfu/testis, but in the absence of detectable markers of apoptosis even 4 days after infection i.e. 1 day later than apoptotic markers were detected in testes infected with $4x10^8$ pfu/testis. Some of the expression of Hif-1α in the testes infected at both doses may be related to invasion by immune cells, as described below. Macrophages and neutrophils both rely on glycolysis to generate energy in the hypoxic conditions associated with inflamed and damaged tissues and Hif-1α also regulates components of the glycolytic pathway (Semenza et al., 1994). Therefore some expression of HIF-1α in infected testes could be associated with immune cells, as well as the GC.

The gap junctions, marked by Cx43, appear more sensitive to adenoviral infection than ectoplasmic specialisations, assessed by espin expression. Connexin43 was not detected in testes infected with $4x10^8$ pfu/testis at 3 and 7 days post infection at a time when GC were undergoing apoptosis. The onset of both GC apoptosis and breakdown of gap junctions precede the first time point in the study so it is not possible to determine which occurs first and whether one event causes the other. Spermatogenesis fails in the absence of Cx43 expression as demonstrated in studies using SC-specific knockout of Cx43 (Brehm et al., 2007) and transplantation of testes from Cx43 deficient mice into wildtype mice (Roscoe et al., 2001). Development of the testes occurred in the absence of Cx43 in these models which may account for the deficiencies in spermatogenesis. Batias et al used mice with impaired spermatogenesis (*ebo/ebo* and *jun-d*$^{-/-}$) to demonstrate that absence of GC caused reduced expression of Cx43 in seminiferous tubules compared to expression in wild-type animals (Batias et al., 1999). Connexin43 expression in these animals was modulated specifically by the elongated spermatids (Batias et al., 1999), but in the virally infected seminiferous tubules with reduced Cx43

expression elongated spermatids had not been lost. In testes from humans with klinefelter's syndrome and mice with mosaic mutation and partial deletion of the Y chromosome similar losses of Cx43 expression were noted (Kotula-Balak et al., 2007). The decline in Cx43 expression in testes infected with adenovirus at $1x10^5$ – $1x10^7$ pfu/testis, was restricted to tubules in which GC cell loss and vacuole formation had already occurred. That Cx43 expression is still present but begins to decline in the absence of GC suggests that in these testes gap junctions begin to breakdown as a consequence of GC loss and not the other way round. The reduced expression may also be due to loss of Cx43 expressed by the GC that are missing as it is reported that Cx43 in the gap junctions is synthesised by both GC and SC (Risley et al., 1992). The reason for complete loss of expression in testes just 3 days after injection with $4x10^8$ pfu/testis, but not in testes infected with lower viral doses, may be a reflection of the more significant damage to the function of the SC caused by the higher doses. Therefore it appears that adenoviral infection may do more than adversely affect the secretion of factors that support GC development by also having an impact on the maintenance of junctional integrity and physical support to the GC.

The ectoplasmic specialisations appeared to be less sensitive to adenoviral infection, because expression of espin was maintained under conditions where Cx43 expression was either lost or diminished. Disturbances in the patterns of expression of espin appeared to occur following altered distribution of SC cytoplasm following GC loss as expression followed the contours of the vacuoles. The loss of espin expression at 7 days following infection with $4x10^8$ pfu/testis may either be a consequence of 1) extensive damage to SC due to the unusually high dose used, as described above, that took longer to become apparent than Cx43 loss or 2) the loss of contact between SC and GC due to the disrupted structure and apoptosis of GC. An *in vitro* study on immortalised SC cultured in the presence or absence of GC detected increased expression of espin protein in SC co-cultured with GC, increase espin expression was not associated with binding between SC and GC (Wolski et al., 2007).

Invasion of immune cells into the interstitium and tubules of testes that had a disrupted architecture consisting of early infiltration by macrophages, at 3 days post-infection, followed by neutrophils by 7 days post-infection. Earlier detection of macrophages than neutrophils would be expected because testes contain resident macrophages in their interstitium. However there is some evidence that the types of macrophages most important for inducing inflammation are not resident in the testis prior to infection, rather they are recruited from the circulating population outside the testis (Gerdprasert et al., 2002). The macrophage marker used in this study does not differentiation between these two populations of macrophages. The infiltration of tubules in these highly damaged testes may occur as a result of disruption of the basement membranes of the tubules as SMA staining at this location also appeared to be disturbed. The suggestion that immune cells enter the testes sequentially is supported by data from EAO experiments in which macrophages were first seen surrounding the perimeter of tubules and then entering the lumen if the tubule were damaged, subsequently there was invasion of the interstitium and lumen by neutrophils (Tung et al., 1970).

In a limited number of tubules in testes infected with $1x10^5$ pfu/testis, neutrophils were observed infiltrating the tubules, and the interstitial macrophage population was slightly enlarged although they did not penetrate into the seminiferous tubules. The infiltration of tubules by neutrophils was less pronounced in the testes injected with $1x10^5$ pfu, probably because the basement of the tubules was relatively intact based upon the distribution of SMA staining. Infiltration of macrophages and neutrophils into seminiferous tubules was not observed following LPS-induced immune response (Gerdprasert et al., 2002), and in that respect the lack of macrophage invasion into tubules in testes infected with $1x10^5$ pfu/testis corresponds to the milder phenotype shared by testes infected with LPS and low doses of adenovirus ($1x10^5$ – $1x10^7$ pfu/testis). The presence of neutrophils in seminiferous tubules and increased numbers of macrophages in the interstitium of testes following infection with $1x10^5$ pfu/testis, but not following infection with $1x10^6$ or $1x10^7$ pfu/testis, could be a consequence of

variation in the viral stocks used or in the way the intra-testicular injections were carried out.

The evidence presented above in relation to gap junctions and ectoplasmic specialisations, suggests that the GC loss observed in adenoviral-infected testes is not a consequence of disruption to Cx43 or espin expression at sites of SC-GC interaction. Involvement of an immune response in the loss of GC from testes injected with less than $1x10^7$ pfu/testis has also not been demonstrated. On this basis, other mechanisms for the impact of adenoviral infection on GC survival should be investigated. Expression of factors essential for support of spermatogenesis by SC, such as activin, steel factor and GDNF, would be obvious first candidates for investigation in testes infected with adenovirus.

Damage to seminiferous tubules associated with intra-testicular injection of adenovirus could be due to another cause. Ogawa et al. reported increased internal testicular pressure resulting in ischemia caused by injections into the efferent ducts during GC transplantation (Ogawa et al., 1997). However the volumes used in that study were between two- and three-fold higher than in my studies. The differences in amount of damage observed when testes were injected with varying concentrations of viral constructs in the same volume of vehicle (trypan blue and saline solution) suggest that the viral concentration had a substantial impact on the damage caused to the tubules, independent of injection of fluids. Other factors that may contribute to tubule damage in addition to the effect of viruses could include the increased pressure in testes described above or an effect of the components of the injection vehicle. The impact of the injection process can not be determined from the present studies because sham operations in which the testes of the mouse would be injected with saline or vehicle (saline solution plus trypan blue) were not carried out. Such sham experiments would determine whether injection of 50 µl of fluid or the presence of trypan blue cause tubule disruption independent of introduction of the adenovirus.

4.4.3 Conclusions

Although adenoviral particles specifically infect SC *in vitro* and *in vivo* we were not able to develop an *in vivo* method for disruption of specific gene expression within SC using this method of gene delivery under normal physiological conditions. This was because although the viral infection resulted in SC-specific expression even modest levels of expression of the reporter protein was associated with significant adverse effects on the integrity of the seminiferous tubules. The adverse effects were present at all doses that achieved substantial transgene expression, and therefore the use of adenoviruses to introduce RNA interference into SC *in vivo* was shown to be unfeasible in this first phase of the study. To achieve SC specific knockdown, methods such as the SC-specific promoter linked to an RNAi construct, or the Cre-lox system described in the introduction will need to be employed. In contrast, adenoviral constructs can be used *in vitro* as vectors, free from the complications encountered *in vivo*.

5 Gene expression in rat testes: studies using Leydig cell depletion with ethane dimethane sulphonate

5.1 Introduction

Steroid action is essential for successful spermatogenesis, and of particular importance in the testis are the actions of androgens. The requirement for SC to express functional AR and respond to androgen signalling has been confirmed by transgenic approaches. Studies on SC function have involved both *in vitro* and *in vivo* models. *In vitro* models used include isolated primary cells and immortalised cell-lines (Denolet et al., 2006b; Mather, 1980; Peschon et al., 1992; Sneddon et al., 2005; Steinberger and Jakubomaik, 1992), such as the SK11 line used in chapter 3. *In vivo* methods used in investigation of androgen response throughout the reproductive tract include models with diminished AR expression (ARKO, SCARKO, *Tfm* mice) or disruption to androgen secretion (steroid implants, *Hpg* mouse, hypophysectomy, and EDS-treatment) (Bartlett et al., 1986; De Gendt et al., 2004; Denolet et al., 2006a; El Shennawy et al., 1998; O'Donnell et al., 1994; Singh and Handelsman, 1995). In chapter 4, a pilot study for a novel method intended for disruption of AR expression was undertaken, however the results were unsatisfactory. As a consequence of abandoning the *in vivo* model in chapter 4, an alternative system employing the testicular toxin ethane dimethanesulphonate (EDS) to induce withdrawal of testosterone in rats, was adopted.

Cycles of spermatogenesis in rat seminiferous tubules are divided into 14 stages (Leblond and Clermont, 1952a). Androgen receptor expression in SC is highest during stages VII and VIII, and expression in LC and PTM does not vary in relation to stages of associated tubules (Bremner et al., 1994). In models in which intratesticular testosterone (ITT) is depleted, including Hypogonadal (*Hpg)* mice and rodents treated with high concentrations of testosterone and oestradiol (T+E) implants, two androgen dependent stages in spermatogenesis have been identified, the first being completion of meiosis and the second conversion of round to elongate spermatids in the process of spermiogenesis

(Hill et al., 2004; O'Donnell et al., 1994; Singh and Handelsman, 1995). These steps are associated with stage VII-VIII tubules, and so these stages have been characterised as 'androgen-dependent' (Sharpe, 1994). Spermatogenesis could be rescued in these models by treatment with high doses of testosterone to restore ITT concentrations (El Shennawy et al., 1998; Franca et al., 1998; Hill et al., 2004; O'Donnell et al., 1994; Singh and Handelsman, 1995).

In the *Hpg* mouse model described above, animals have developed in the absence of testosterone and gonadotrophins, with impacts on testis size, proliferation of cells, and size of cell populations within the testis (Singh and Handelsman, 1995). A single injection of EDS diminishes testosterone within 6 hours of treatment and totally ablates mature LC from the interstitium within 3 days in adult rats. Partial recovery of serum testosterone levels occurred after 14 days, a graph of serum testosterone over a 10 week period is shown in Figure 5-1 (Bartlett et al., 1986). Use of EDS to disrupt LCs and cause depletion of ITT can be applied to rats, guinea pigs, and Syrian hamsters but not to mice. In mice spermatogenesis is disrupted, but not by disruption of LC which are resistant to effects of EDS (Kerr et al., 1987).

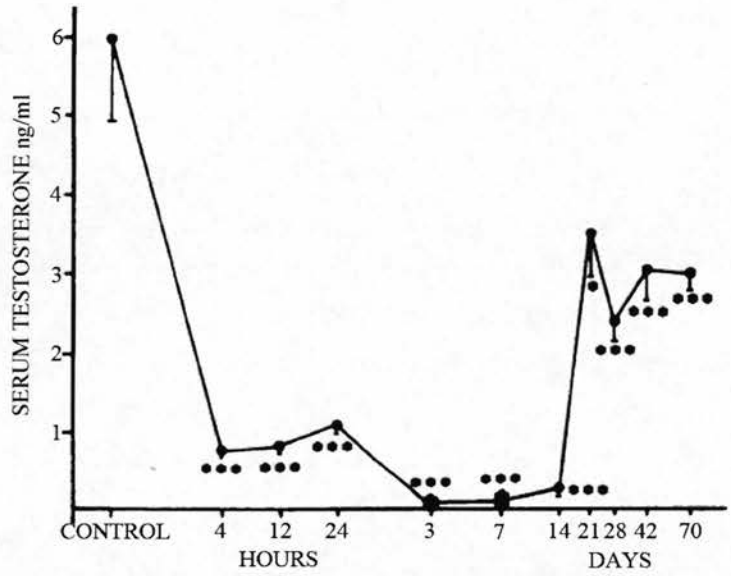


Figure 5-1: Serum testosterone detected in an adult rat between 4 hours and 70 days after a single injection of EDS. Plotted values show mean ± standard error, * $p<0.025$, ** $p<0.01$, *** $p<0.001$ compared to control. Adapted from (Bartlett et al., 1986).

Disruption to spermatogenesis and the seminiferous tubule in response to a single EDS injection occurs 3 days after EDS-treatment with degeneration of a minority of pachytene spermatocytes at stages VII and VIII, indicating high androgen-sensitivity during these stages of the epithelial cycle and for the development of these cells (Bartlett et al., 1986; Kerr et al., 1993a). Analysis of gene and protein expression within 6 days of EDS-treatment can be performed on whole testes because the majority of the GC complement is maintained so results are not skewed by altered cell composition within the testis (Turner et al., 2001), although absence of testosterone's impact may alter GC gene expression influencing expression in whole testis. A week after EDS-treatment, abnormal development of spermatocytes and spermatids in stage VII-XI tubules, particularly stage 7 and 8 spermatids, plus retention of stage 19 spermatids was observed (Bartlett et al., 1986; Kerr et al., 1993a). Sertoli cells are also affected and develop vacuoles in the basal region 7 days after EDS-treatment which are thought to be caused

by interference with tight junctions due to androgen-deficiency (Bartlett et al., 1986). Disruption to GC from stage VII tubules and appearance of vacuoles can be prevented by testosterone replacement (Kerr et al., 1993a). Expression of AR protein, and its nuclear localisation has been shown to be dependent upon androgen signalling in numerous androgen depletion models including in EDS-treated rats (Bremner et al., 1994; Hill et al., 2004). In the testes of rats treated with EDS for 6 days, immunodetection of AR protein expression is completely lost from SC, PTM, and any remaining or newly formed LC, following depletion of intra-testicular testosterone (Atanassova et al., 2006; Bremner et al., 1994). Immunopositive staining for AR was reinstated by treatment with exogenous testosterone, the pattern of differential expression between tubules at different stages was indistinguishable from that of untreated rats (Bremner et al., 1994; Turner et al., 2001).

The EDS model has been used to demonstrate that secretion of seminiferous tubule fluid is stage dependent; secretion in tubules at stages VI-VIII is 2-fold greater than in tubules at other stages. Differential secretion is absent in tubules of rat testes between 3 and 6 days after treatment with EDS, secretion in stage VI-VIII tubules is equal to that at stages II-V and IX-XII. Increased secretion in stage VI-VIII tubules can be reinstated by treatment with testosterone (Sharpe et al., 1994; Sharpe et al., 1992). Androgens regulate the secretion but not synthesis of tubule fluid proteins. Secretion of constitutively expressed SC proteins such as SGP-1 and -2 is not regulated by androgens, but secretion of cyclic protein 2 (CP-2) and proteins secreted by GC and SC via regulated pathways are affected (McKinnell and Sharpe, 1995).

In recent experiments androgen responsive genes have been identified whose mRNA expression has been assessed through arrays and quantitative RT-PCR on testes of SCARKO and anti-androgen-treated mice. Androgen responsive genes identified in addition to *AR*, include *Rhox5* (a reproductive homeobox gene), *β3-tubulin*, *espin*, and *tight junction protein 1* (Abel et al., 2008; Denolet et al., 2006a). Array data supported by immunohistochemistry and Western blotting on tissues derived from another SC-

specific AR knockout mouse model (*Ar*$^{flox(ex1\text{-}neo)}$; *Amh-cre*) demonstrated androgen-dependent expression of the tight junction component *claudin3* in SC (Meng et al., 2005).

There is extensive intimate contact between SC and GC in seminiferous tubules through ectoplasmic specialisations, gap-junctions, tubulobulbar complexes and, desmosome-like junctions (McGinley et al., 1979; Russell, 1977a; Russell, 1977b; Russell and Clermont, 1976) Holdcraft et al. reported androgen-sensitivity of spermiation and adhesion of round spermatids prior to elongation, suggesting that the primary role of androgen signalling is to influence adhesion between SC and GC (Holdcraft and Braun, 2004). Therefore expression of other genes in response to androgens has also been examined, with particular attention on expression of junctional proteins within seminiferous tubules. In rats treated with high doses of testosterone and oestrogens to induce chronic reduction in intra-testicular testosterone, stage 8 round spermatids detach from the seminiferous epithelium, however the integrity of ectoplasmic specialisations at the sites of spermatid loss, determined by espin expression, is maintained (O'Donnell et al., 1994). *Ar*$^{flox(ex1\text{-}neo/y)}$; Amh-Cre mice share a similar pattern of GC loss, and the permeability of the BTB in these mice is increased in association with a loss of *claudin3* expression, however tight junctions and expression of their components, such as *claudin11* and *occludin*, are maintained (Holdcraft and Braun, 2004; Meng et al., 2005). Staining for espin at basal ES and N-cadherin are also reported to be unchanged following suppression of testosterone and FSH (Beardsley and O'Donnell, 2003). In contrast to Beardsley et als findings, subsequent studies report increased expression and a wider distribution of N-cadherin within tubules of testosterone-depleted animals. Increased N-cadherin distribution was attributed to dissociation from β-catenin and disruption to adherens junctions (Xia et al., 2005). Testosterone depletion also induces increased expression of the tight junction adaptor protein, zona occludins-1 (ZO-1), but its distribution within the TJ was maintained as was BTB integrity (Xia et al., 2005). Connexin43 (Cx43) is a component of gap junctions identified in tissues including the testis. Within the testis Cx43 is expressed in junctions between adjacent SC (Risley et

al., 1992; Tan et al., 1996) and in the adluminal compartment between SC and GC (Batias et al., 1999; Tan et al., 1996). Expression of Cx43 is stage-specific in rat and mouse testes. In rat testes, little or no positive staining for Cx43 was seen in stages IX-XIV whilst strong staining was present in tubules at stages I-VIII. In mice, strong staining was restricted to stages VI-VIII, stages I-V and IX-XII exhibited low expression (Risley et al., 1992).

5.1.1 Aims

To use the established EDS-treated rat model to validate androgen-responsive expression of target genes identified *in vitro* and *in vivo*, using models including SCARKO mice and rodents with depleted intra-testicular testosterone. Also, extend studies in previous array-based publications by investigating protein expression. Investigations focus on the established androgen responsive genes, *AR* and *Rhox5*, and on components of junctions or the cytoskeleton. These have either been described as androgen-responsive in previous studies or are related targets with potentially for novel androgen-responsive expression.

5.2 Materials and Methods

5.2.1 Animal treatments

Treatment of rats with EDS and testosterone esters was undertaken as described by Turner et al. (Turner, Morley et al. 2001). Briefly, adult male Wistar rats were treated with 75 mg/kg ethane dimethane sulphonate (EDS, synthesised in house) in dimethylsulphoxide/water (1:3; v/v) by intra-peritoneal injection 6 days prior to testes being collected. A second group received the same EDS injection plus a simultaneous subcutaneous injection with 25 mg/kg testosterone esters (TE; Organon Laboratories, Cambridge, UK) in arachis oil and a second TE injection 3 days later, testes were collected 6 days after the initial EDS treatment. A third (vehicle control) group of adult rats were injected with dimethylsulphoxide/water (1:3; v/v) by intra-peritoneal injection 6 days prior to testes being collected. EDS treatment depletes serum and testicular testosterone to undetectable levels (by RIA) by destroying Leydig cells, this treatment also depletes oestrogens for which testosterone is a precursor. TE injections compensate for the reduced testosterone levels caused by Leydig cell ablation. All treatments were carried out by Prof Richard Sharpe, of the Human Reproductive Sciences Unit Edinburgh, under his project licence. I am most grateful to Prof Sharpe for allowing me to use these tissues. Effectiveness of the EDS-treatment was confirmed at the time of testicular tissue collection by checking for reduced prostate size, and efficiency of rescue by testosterone esters was established by the prostates returning to a similar size to that of control animals (Turner et al., 2001).

5.2.2 TaqMan Q-RT-PCR

Samples of RNA extracted from the testes of EDS-treated and control adult rats were incubated in a random hexamer primed reverse transcriptase reaction to produce cDNA (section 2.3.3.1). The cDNA was used in Universal Human Probe Library™ (section 2.3.3.4.) TaqMan reactions that were undertaken on an ABI 7900 HT Real-Time PCR Machine.

The UPL TaqMan primer and probe sequences designed to quantify rat mRNA transcripts are shown in Table 5-1.

Table 5-1: Universal Human Probe Library™ primer and probe sequences for TaqMan Q-RT-PCR of rat transcripts.

Gene	Forward Primer 5' – 3'	Reverse Primer 5' – 3'	Probe (Probe number)
AR	TTCTTCTTCT CTGCCTCTTTTACC	AAGAAATACAACAA AACTCAAAACACA	GGCTCCTG (#92)
β3-tubulin	CAGAGCCAT TCTGGTGGAC	GCCAGCACC ACTCTGACC	GAGCCTGG (#116)
Espin	TGCAGTG GCTCACAC	GACCGTGGCA CCAGAGTTAT	GGTGGCTG (#83)
Rhox5	AGCGCATT TTGCTAAGCAGT	TCCATCCATCT ATCAAGCTCCT	TCCTCCAG (#65)

5.2.3 Western blotting

Western blots were performed on proteins extracted from rat testis tissue using 1x RIPA (section 2.4.1). The rat testes were from animals treated with EDS, EDS plus TE, or injected with vehicle (control) (section 5.2.1). Western blots were performed as described in section 2.4.2, and protein bands were visualised on membranes using fluorescence (LiCor). Blotting was carried out on rat tissues for β-tubulin and β3-tubulin, junctional proteins, and Rhox5. The primary antibodies used are listed in Table 5-2. The secondary antibodies used are described in

Table 2-5 (section 2.4.2).

Table 5-2: Primary antibodies for Western blots.

Antigen	Dilution	Species Raised	Source
AR	1:200	rabbit	US Biological
β-Tubulin	1:200	mouse	Sigma
β3-Tubulin	1:400	mouse	Sigma
Espin	1:5000	mouse	Transduction laboratoriess
Rhox5	1:1500	Rabbit	Abcam
β-Actin (Loading control	1:5000	mouse	Sigma
β-Actin (Loading control)	1:1000	rabbit	Abcam

5.2.4 Immunohistochemistry

Immunohistochemistry was performed using both DAB and fluorescent staining methods (sections 2.5.2 and 2.5.3). The rat testis sections were stained for junctional proteins, Sertoli cell markers (Sdmg-1, GATA-1), androgen responsive product (Rhox5) and PTM cell marker (SMA). The primary antibodies used are shown in Table 5-3.

Table 5-3: Primary antibodies for immunohistochemistry.

Antigen	Dilution	Species raised	Source
AR	1:50	rabbit	Santa Cruz
β-tubulin	1:1500	Mouse	Sigma
Cx43	1:100	Rabbit	Zymed
Espin	1:30	Mouse	Transduction Laboratories
N-cadherin	1:7000	mouse	Zymed
Sdmg-1	1:1000	Rabbit	Gift from Ian Adams (MRC HGU, Edinburgh)
SMA	1:500	mouse	Sigma
ZO-1	1:100	Rabbit	Zymed

5.3 Results

5.3.1 mRNA analysis

5.3.1.1 AR

Expression of *AR* mRNA was quantified by Taqman quantitative PCR in samples extracted from rat testes treated with EDS or EDS plus testosterone esters (Figure 5-2). Treatment with EDS to ablate LC and reduce intra testicular testosterone reduced expression of *AR* to less than 30% of control expression, and testosterone replacement with testosterone esters in parallel with EDS-treatment increased expression to over double that seen in controls. Although the differences in *AR* expression were large, the small number of animals in each treatment group (n=3 in control, and n=4 in EDS and EDS plus T) meant that the effects of treatments were not significant when analysed by Kruskal-Wallis test, p=0.49. Wide variation in expression between animals within each group compounded the effect of small sample size.

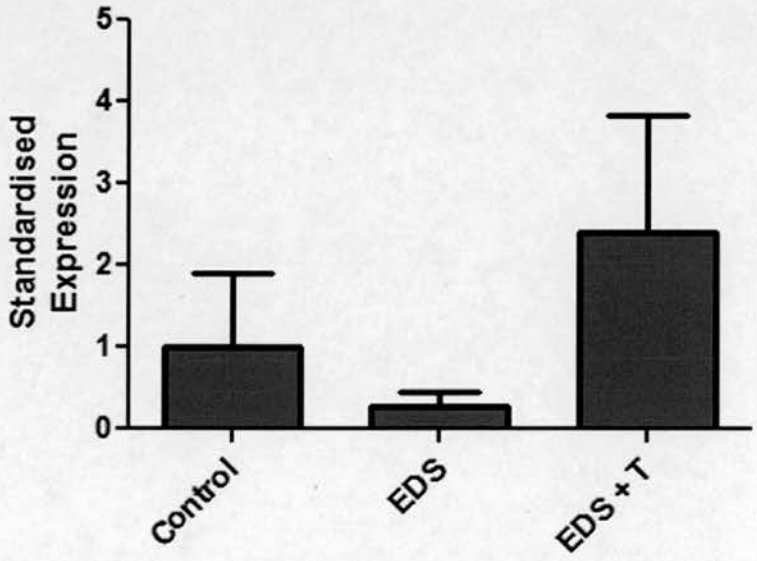


Figure 5-2: Quantification of *androgen receptor* mRNA in rat testes by Taqman Q-RT-PCR. Testes were collected 6 days after treatment with EDS or EDS plus TE. Expression was standardised to that of control rat testes. Bars represent average expression ± SEM, for n=4 animals per treatment (except for control group with n=3 animals). A Kruskal-Wallis test demonstrates no significant difference (p=0.49) in expression between treatments.

5.3.1.2 Rhox5

Expression of *Rhox5* mRNA was also quantified by Taqman RT-PCR (Figure 5-3). Treatment of rats with EDS caused almost total depletion of *Rhox5* mRNA to less than 1.5% of control levels. Expression of *Rhox5* was maintained in rats treated with EDS and testosterone esters. Although *Rhox5* mRNA was almost undetectable in the EDS-treated group the small numbers of repeats in each treatment group (n=2 in control group, and n=4 in EDS and EDS plus T) means that the effect of treatments on *Rhox5* expression are not significant when assessed by Kruskal-Wallis test, p=0.42. High variability of expression within treatment groups, particularly control animals, compounded the impact of the small numbers of animals in each treatment group.

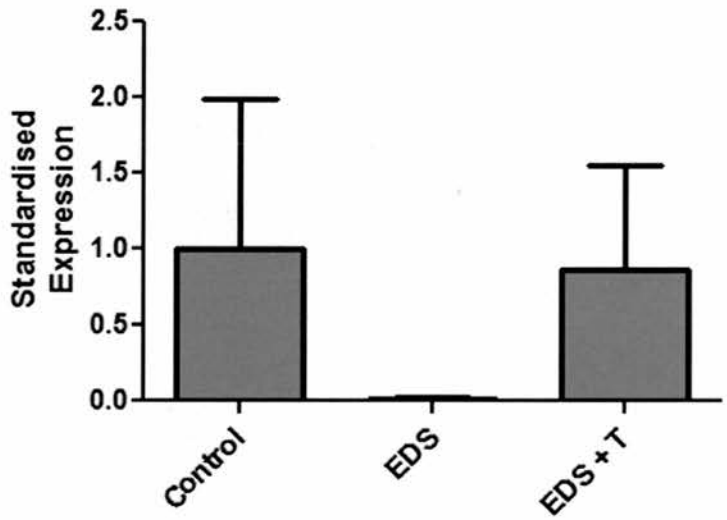


Figure 5-3: Quantification of *Rhox5* expression in testes of rats treated with EDS or EDS plus TE by Taqman. Testes were collected 6 days after treatment, and expression standardised to that of untreated control testes. Bars represent average expression ± SEM, for n=4 animals per treatment (except for control group with n=2 animals). A Kruskal-Wallis test demonstrates no significant difference (p=0.42) in expression between treatments.

5.3.1.3 β3-tubulin

Expression of *β3-tubulin* mRNA in the testes of rats treated with EDS was depleted to less than 3% of expression detected in untreated (control) rats' testes. Expression was increased to 140% of control level when intra testicular testosterone loss was compensated for with testosterone ester treatment. Expression was quantified by Taqman quantitative RT-PCR, and is presented standardised to expression in control testes (Figure 5-4). Depletion of *β3-tubulin* mRNA in testes of EDS-treated rats is almost absolute, but the small number of animals in each treatment group (n=2 control animals, and n=4 in EDS and EDS plus T groups) resulted in the Kruskal-Wallis test finding no statistically significant effect of the treatments given, p=0.44. The impact on statistical significance of high variability of *β3-tubulin* expression within the EDS plus testosterone treatment and control groups was enhanced by the small numbers of animals in each treatment.

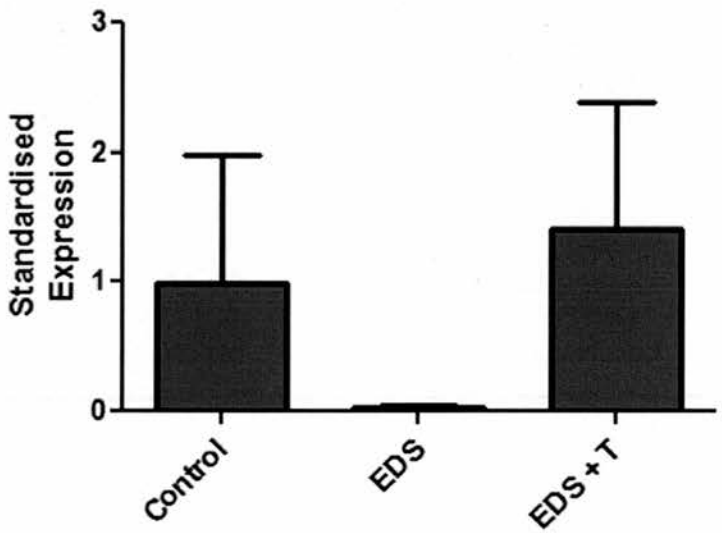


Figure 5-4: Taqman quantification of *β3-tubulin* expression in rat testes treated with EDS or EDS plus TE. Testes were collected 6 days after treatment, and expression standardised to that of untreated control testes. Bars represent average expression ± SEM, for n=4 animals per treatment (except for control group with n=2 animals). A Kruskal-Wallis test demonstrates no significant difference (p=0.44) in expression between treatments.

5.3.1.4 Espin

Expression of *espin* mRNA in testes of rats treated with EDS is reduced to less than 20% of expression detected in testes of untreated (control) animals. Treatment with testosterone esters in addition to EDS causes expression of *espin* mRNA to increase by 50% compared to control rat testes. A graph of average expression and associated error bars is shown in Figure 5-5. Although the changes in mRNA expression are dramatic in response to EDS or EDS plus testosterone ester treatments, the analysis of expression in small group sizes (n=3 in control group, and n=4 EDS and EDS plus T animals) meant that treatments were found not to have a significant effect on *espin* expression, p=0.49. The large variation in expression detected in the testes of control and EDS plus testosterone ester treated animals was also responsible for the lack of significance, and its impact was inflated by the small group sizes.

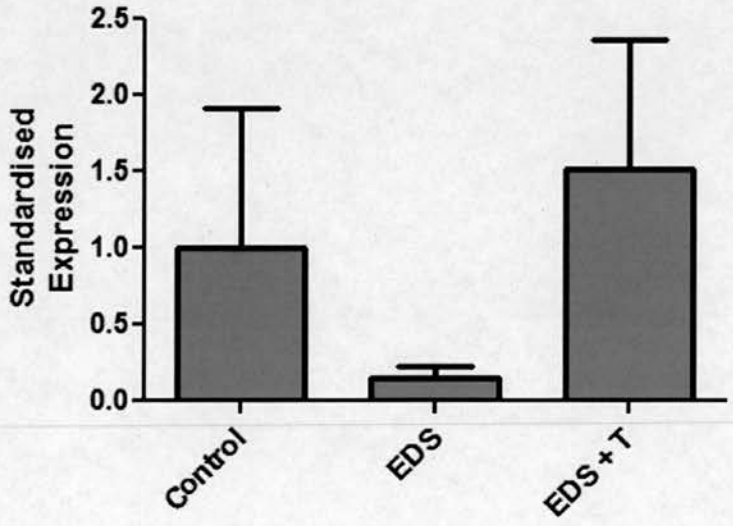


Figure 5-5: Quantification of *espin* expression in EDS- or EDS plus TE treated rat testes. Expression was assessed in testes 6 days after treatment by Taqman, and expression standardised to that in untreated (control) testes. Bars represent average expression ± SEM, for n=4 animals per treatment (except for control group with n=3 animals). A Kruskal-Wallis test demonstrates no significant difference (p=0.49) in expression between treatments.

5.3.2 Western analysis

5.3.2.1 AR

Expression of AR protein in testes of rats was quantified by fluorescent Western blotting (Figure 5-6). Panel A shows the fluorescently labelled protein bands of AR (110 kDa, green) and the loading control β-actin (42 kDa, red). Expression of AR was detected in all testicular samples, but differences in expression level between treatment groups were not immediately apparent. By measuring the intensity of fluorescence associated with AR and β-actin in each well the expression of AR, standardised to β-actin, was quantified for each sample. The mean AR expression in testes from each treatment group (n=4) is shown in panel B, expression is presented relative to average expression in testes of control animals. A significant effect of treatments on AR expression was found overall ($p=0.05$) using the Kruskal-Wallis test, and Dunn's multiple comparison post-hoc test revealed that expression was significantly reduced ($p<0.05$) specifically in testes of EDS-treated rats compared to EDS plus TE treated rats. Expression in control and EDS plus TE testes was relatively similar.

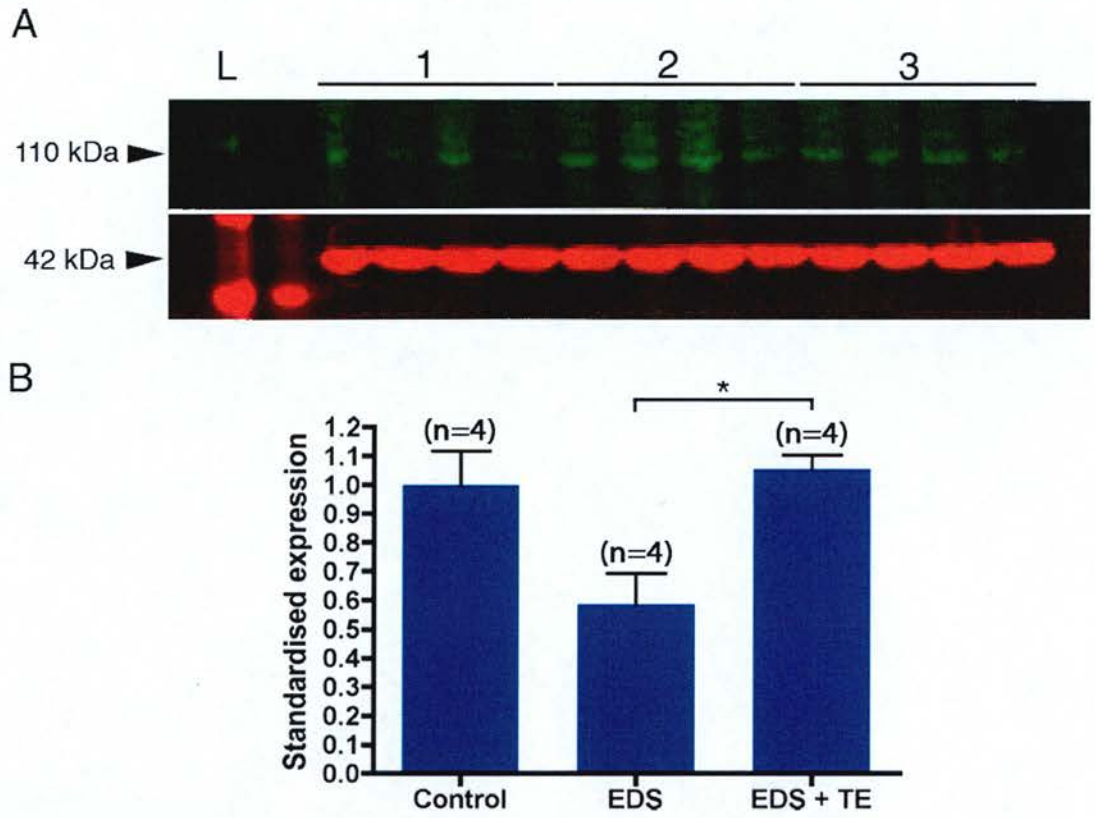


Figure 5-6: Expression of total AR protein in testes of rats treated with EDS, EDS plus TE or vehicle (control). Panel A shows the fluorescently-labelled protein bands on the Western membrane associated with AR (110 kDa, green) and β-actin (42 kDa, red), lane L contains seeBlue ladder. The lanes contain protein from rats treated with EDS (1), EDS plus TE (2), and control rat testes (3). The graph in panel B shows the expression of AR quantified from the Western in panel A. AR expression was standardised to β-actin in each well, and the mean AR expression ± SEM in each treatment group (n=4) is presented relative to expression in control testes injected with vehicle. Statistical analysis (Kruskal-Wallis test) reported a significant effect due to treatment ($p= 0.05$). *; $p<0.05$.

5.3.2.2 Rhox5

Total expression of Rhox5 in testicular samples, as evaluated by Western blot, is shown in Figure 5-7. Panel A shows the fluorescence associated with Rhox5 and β-actin bands on the Western membrane, panel B shows the quantification of protein expression from measuring the fluorescence of the bands. Protein expression was assessed in four

samples for each treatment. The Rhox5 (30 kDa) expression in each sample was standardised to the associated β-actin (42 kDa) expression in the same lane, and presented relative to the level in control tissues. Expression of Rhox5 in the testes of EDS-treated rats was ~20% less than in control animals, and treatment with EDS plus TE reduced expression by ~45%. Although expression was decreased by treatment with EDS and EDS plus TE, the changes were not significant ($p=0.12$, Kruskal-Wallis test).

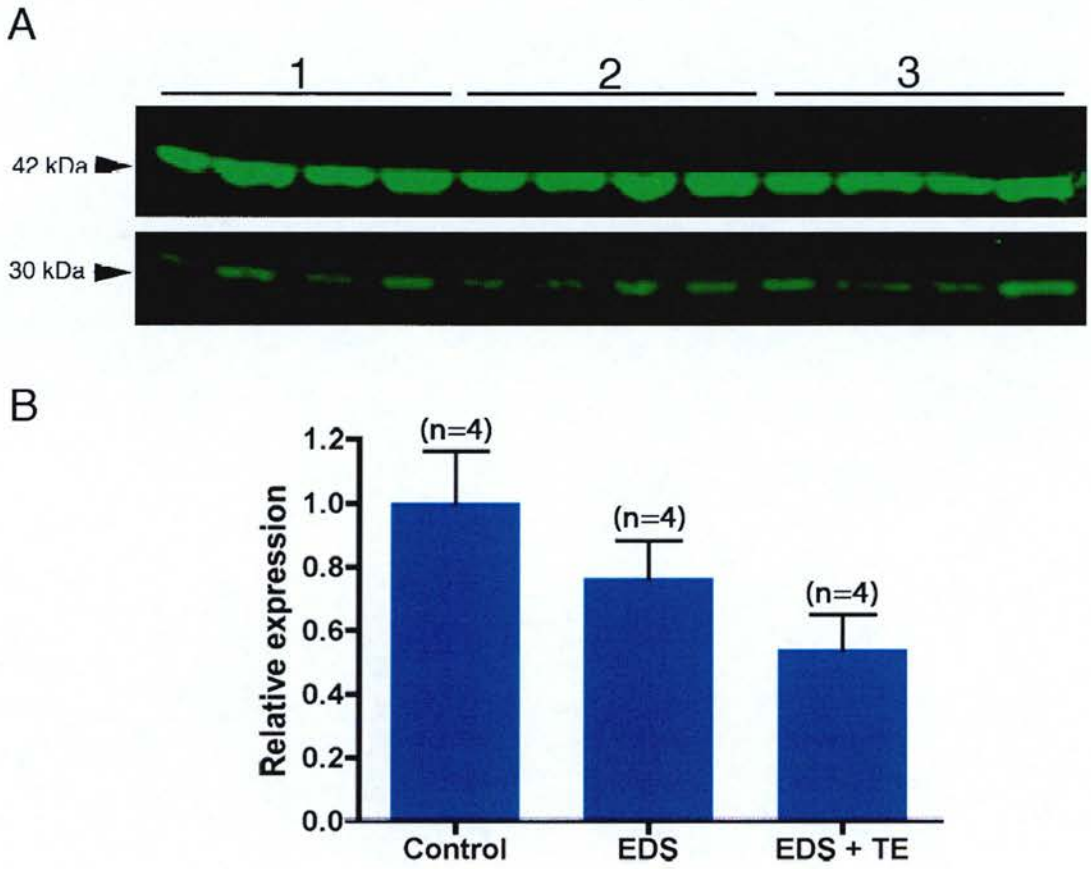


Figure 5-7: Expression of Rhox5 protein expression in the testes of adult rats treated with EDS or EDS plus TE. Panel A shows expression of β-actin (top row) and Rhox5 (bottom row) in the testes of adult rats treated for 6 days with EDS (1) or EDS plus TE (2), or injected with vehicle (control, 3). Panel B shows the standardised Rhox5 expression (Rhox5/β-actin expression) for each treatment relative to expression in the testes of animals injected with vehicle (control). The graph shows mean ± SEM in n=4 samples/treatment. Non-parametric statistical analysis by Kruskal-Wallis test reported no significant difference between treatments ($p = 0.12$).

5.3.2.3 β3-tubulin

Total expression of β3-tubulin protein in rat testes treated with EDS, EDS plus TE, or injected with vehicle (control) is presented in Figure 5-8. Panel A shows the

fluorescently-labelled β3-tubulin and β-actin on a membrane, whilst panel B shows the average expression of β3-tubulin normalised to β-actin within the same lane, quantified from 4 animals/treatment. The β3-tubulin expression in the EDS and EDS plus TE samples were standardised to expression in control testes, given a value of 1. There was substantial expression of β3-tubulin protein in all samples. Expression in EDS-treated testes was ~10% greater than in control samples, but expression in EDS plus TE samples was ~30% lower than control testes. Despite these differences no statistically significant difference between the 3 groups was reported ($p=0.23$).

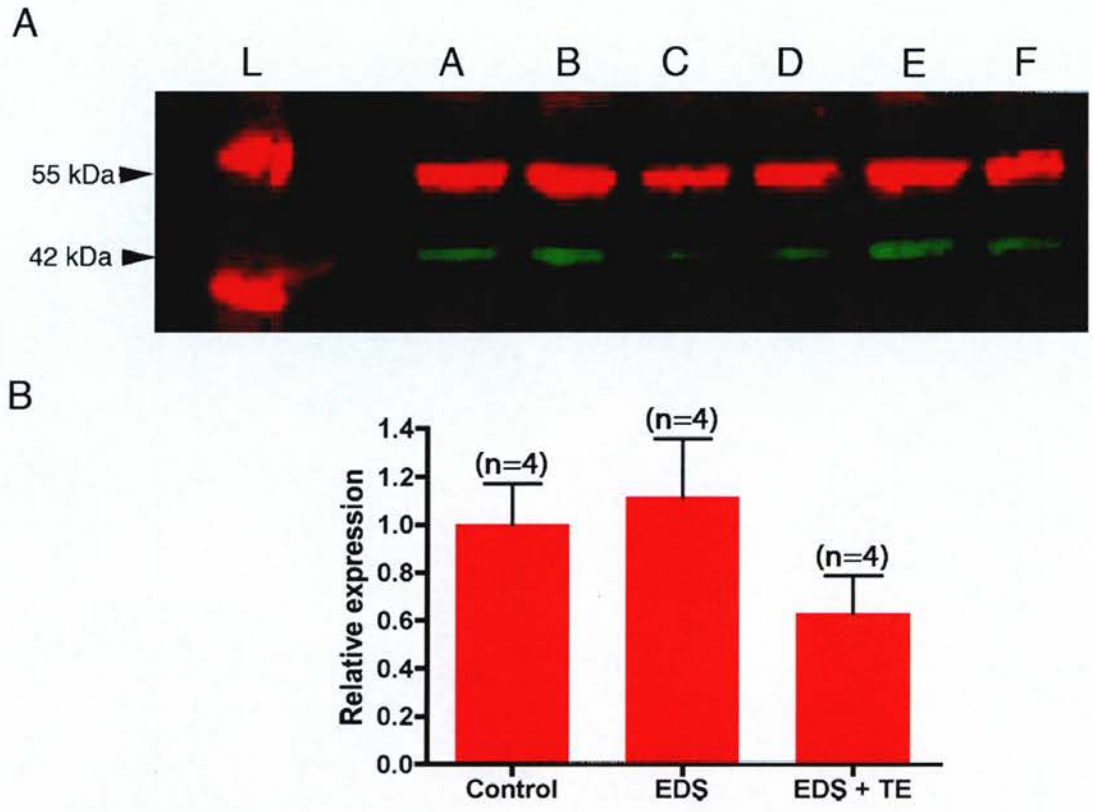


Figure 5-8: Expression of β3-tubulin protein expression in the testes of adult rats treated with EDS or EDS plus TE. Panel A shows expression of β3-tubulin (red) and β-actin (green) in the testes of adult rats treated for 6 days with EDS (A & D), EDS plus TE (B & E), or injected with vehicle (C & F, control). On the left of the image (lane L) is the Seeblue protein ladder. Panel B shows the standardised β3-tubulin expression (β3-tubulin/β-actin expression) for each treatment relative to expression in the testes of control animals injected with vehicle. The graph shows mean ± SEM for n=4 samples. Kruskal-Wallis non-parametric analysis revealed no significant difference between treatments ($p = 0.23$).

5.3.2.4 β-tubulin

Whilst expression of the β3 isoform of β-tubulin was quantified with a specific antibody (Figure 5-8), the total expression of all β-tubulin isoforms was assessed using a pan-β-tubulin antibody (Figure 5-9). Panel A shows the fluorescently labelled protein bands of β-tubulin and β-actin, and panel B is the quantification of fluorescence from Western blots on 4 samples / treatment. The quantification presented is the average β-tubulin

expression normalised to β-actin within the same lane for each treatment, and presented as a proportion of the expression in control testes. Quantification showed that expression of β-tubulin in the testes of EDS and EDS plus TE treated rats was not significantly different from controls.

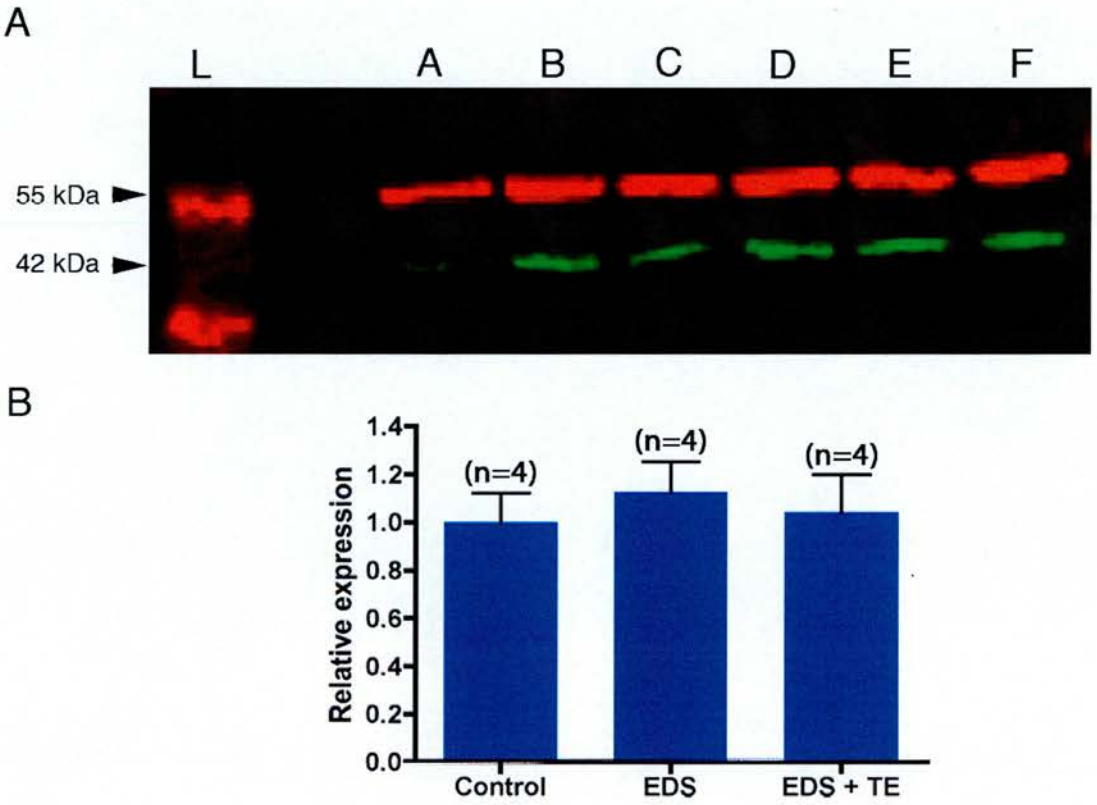


Figure 5-9: Expression of β-tubulin protein expression in the testes of adult rats treated with EDS or EDS plus TE. Panel A shows expression of β-tubulin (red) and β-actin (green) in the testes of adult rats treated for 6 days with EDS (A & D), EDS plus TE (B & E), or injected with vehicle (C & F). On the left of the image (lane L) is the Seeblue protein ladder. The panel B shows the standardised β-tubulin expression (β-tubulin/β-actin expression) for each treatment relative to expression in the testes of control animals injected with vehicle. The graph shows mean ± SEM for n=4 samples. Non-parametric statistical analysis by Kruskal-Wallis test demonstrated no significant difference between treatments ($p = 0.74$).

5.3.2.5 Espin

Fluorescent Western blotting was employed to visualise and quantify expression of espin protein in the testes of rats injected with vehicle and those treated with EDS or EDS plus TE (Figure 5-10). Panel A shows the fluorescently stained espin and β-actin on the Western membrane, panel B shows the quantification of average espin expression in each treatment. The espin expression presented in the graph is based upon 4 animals per treatment and was normalised to β-actin expression within the same lane and standardised to expression in the control testes (given a value of 1). Substantial expression was seen in all treatments; there was no significant difference in espin expression between any of the treatments (p=0.60).

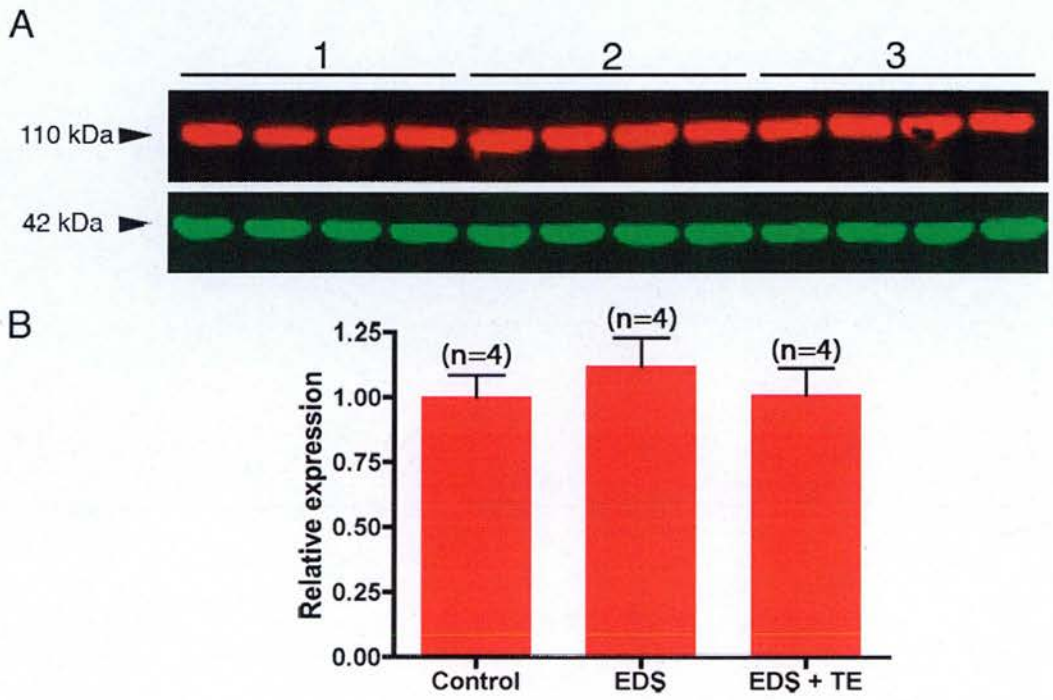


Figure 5-10: Expression of espin protein in the testes of adult rats treated with EDS or EDS plus TE. Panel A shows expression of espin (red) and β-actin (green) in the testes of adult rats injected with vehicle (1) and of adult rats treated for 6 days with EDS (2) or EDS plus TE (3). Panel B shows the standardised espin expression (espin/β-actin expression) for each treatment relative to level in the testes of control animals injected with vehicle. The graph shows mean ± SEM for n=4 samples. Kruskal-Wallis non-parametric analysis showed no significant difference between treatments (p = 0.60).

5.3.3 Immunohistochemistry

5.3.3.1 Sdmg-1

Sdmg-1 is a marker of SC cytoplasm expressed at all stages of the spermatogenic cycle and was visualised using DAB immunohistochemistry in rat testes (Figure 5-11). As expected, expression of Sdmg-1 was not altered by treatment with EDS with or without TE. Positive expression is limited to within the seminiferous tubules and was clearly localised to SC cytoplasm passing between the GC populations resulting in a pattern of narrow bands running from the basement of the tubules through the epithelium towards the lumen. In sections collected from rats treated with EDS, mature LC are absent reducing the volume of the interstitium (in Figure 5-11 panels A, B, C and D) but SC cytoplasm did not appear grossly different.

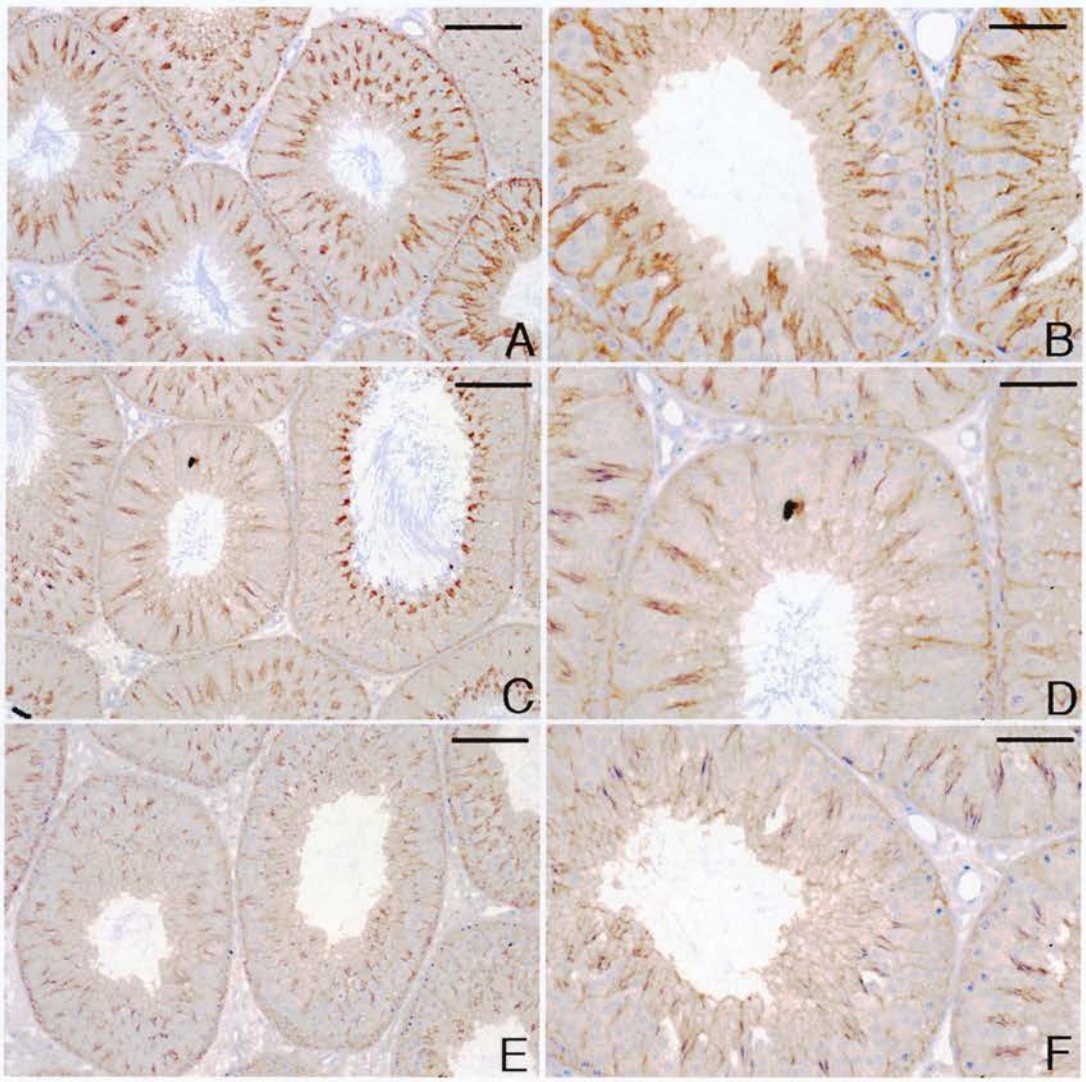


Figure 5-11: Expression of Sdmg-1 in the testes of rats treated with EDS (A & B), EDS plus TE (C & D), or vehicle control (E & F). Testes were recovered after treatment for 6 days, and expression of Sdmg-1 was visualised through DAB immunohistochemistry. Images shown are magnified to x20 (A, C, & E) or x40 (B, D, & F). Scale bars represent 100 μM in x20 and 50 μM in x40 images. Note the absence of mature LC and reduced volume of the interstitium in sections from EDS treated rats.

5.3.3.2 AR

Western blotting for AR detected reduced amounts of AR in the testes of EDS-treated rats compared to in animals treated with vehicle or EDS plus TE (Section 5.3.2.1).

Immunostaining of fixed testes confirmed expression of AR in nuclei of SC, PTM, and LC in control sections (Figure 5-12, panels E and F). Immunopositive LC in control testes are indicated by arrows in panels E and F. AR expression was still detected in SC and PTM of rats treated with EDS and EDS plus TE, there was no obvious change in intensity of immunostaining. As expected, intensity of immunostaining in SC was stage-dependent with most intense staining in sections of tubules at stages VI-VIII (Bremner et al., 1994). In the EDS and EDS plus TE treated animals AR positive cells surrounding blood vessels were present (panel D) and the interstitium contained fewer cells and appeared to comprise a smaller proportion of the sections. Staining was observed in the cytoplasm of elongated spermatids, this is non-specific background staining as it does not occur when other antibodies to AR are used (Bremner et al., 1994).

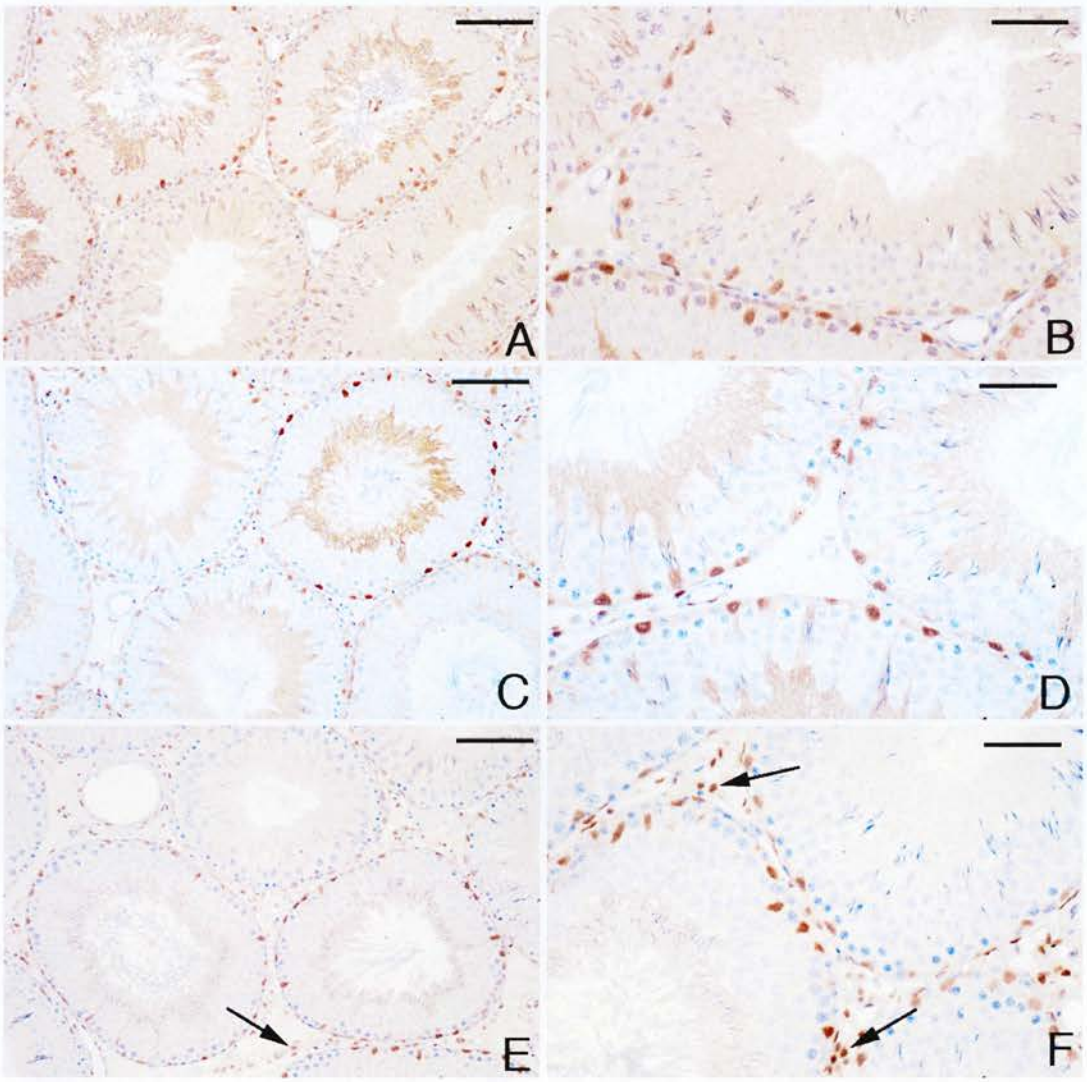


Figure 5-12: Expression of AR in the testes of rats treated with EDS (A & B), EDS plus TE (C & D), or vehicle control (E & F) for 6 days. Expression of AR was visualised by DAB immunohistochemistry. Images presented are magnified x20 (A, C, & E) or x40 (B, D, & F), and error bar represent 100 μM in x20 and 50 μM in x40 images. Immunostained LC in control testes are indicated by arrows in panels E and F.

5.3.3.3 Espin, N-cadherin, and zona occludins -1

Expression of the junctional proteins; espin (in ectoplasmic specialisations, a testis-specific form of adherens junction described in section 1.2.4.2), N-cadherin (another component of ectoplasmic specialisations described in section 1.2.4.2) and zonula occludens-1 (ZO-1, a tight junction associated adaptor protein described in section 1.2.4.1), were localised in the testes of control rats injected with vehicle and rats treated with EDS or EDS plus TE by fluorescent immunohistochemistry (Figure 5-13). Co-expression of espin and ZO-1 are shown in panels A, C and E, and expression of N-cadherin is shown in panels B, D and F, all sections were co-stained with DAPI which highlights the nuclei of cells. Expression of espin was localised with the basal ectoplasmic specialisations (indicated by starred arrow in panel C) at the base of seminiferous tubules as well as the sites of interaction between SC and elongate spermatids closer to the lumen (apical ectoplasmic specialisations, indicated by plain arrows in panels A and E). At the periphery of the seminiferous tubules espin staining was not continuous. ZO-1 expression was also localised to sites at the base of the seminiferous tubules (also indicated by starred arrow, in panel C), which should correspond to tight junctional complexes between SC that are important for integrity of the BTB. Immunopositive staining of N-cadherin was concentrated at the periphery of the tubules (indicated by starred arrow in panel B) and close to the lumen (indicated by plain arrow in panel F). The gross pattern of expression of N-cadherin, espin and ZO-1 was not altered by treatment with EDS or EDS plus TE compared to control testes. Espin and N-cadherin were both localised to apical and basal ES in the seminiferous tubules (indicated by plain and starred arrows in panels respectively), in line with co-expression at these sites. However N-cadherin is also observed in a pattern resembling cytoplasmic staining in SC, in the absence of espin. The 'cytoplasmic' N-cadherin staining (indicated by arrow heads in panels D and F) is weaker than at the apical and basal ES, where very strong staining is observed, and is thought to represent non-specific background.

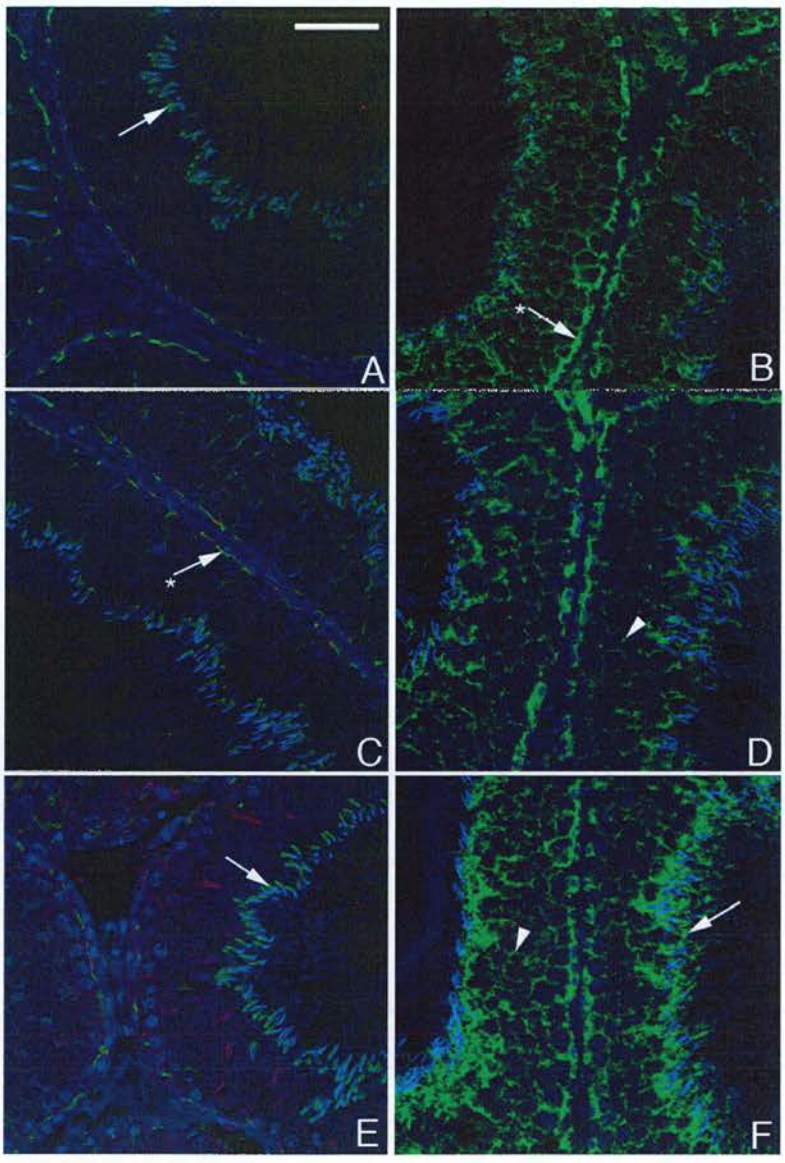


Figure 5-13: Fluorescent immunohistochemistry for expression of espin, ZO-1 and N-cadherin in testes of adult rats treated with EDS or EDS plus TE. Testes of vehicle treated rats (panels A & B) and rats treated for 6 days with EDS (panels C & D) or EDS plus TE (panels E & F) were stained using antibodies specific for espin and ZO-1 (green and red respectively, in panels A, C, & E) and for N-cadherin (panels B, D, & F). Expression at basal locations (BTB and basal ES) is indicated by arrows marked by asterisks. Expression at apical ectoplasmic specialisations is indicated by arrows. Arrowheads alone indicate non-specific 'cytoplasmic' N-cadherin staining. All sections were counterstained with DAPI nuclear stain (blue) and images taken at 40x magnification, the scale bar represents 50 μm.

5.3.3.4 Connexin43

Expression of connexin43 (Cx43), a component of gap junctions, and SMA (PTM cell marker) were visualised by fluorescent immunohistochemistry (Figure 5-14 & Figure 5-15). Expression of Cx43 was localised to sites between SC and sites of SC-GC interaction with prominent staining at the base of the seminiferous tubules (indicated in figures by plain arrows) and near the tubules' lumen where SC and elongate spermatids make contact (indicated by starred arrows in the figures). Expression and localisation of Cx43 were not different in control testes compared with those rescued from EDS or EDS plus TE treated rats. Smooth muscle actin (SMA) was stained to visualise the peritubular myoid cells at the perimeter of the tubules and its expression was not disrupted by EDS or EDS plus TE treatment even though these cells are AR positive.

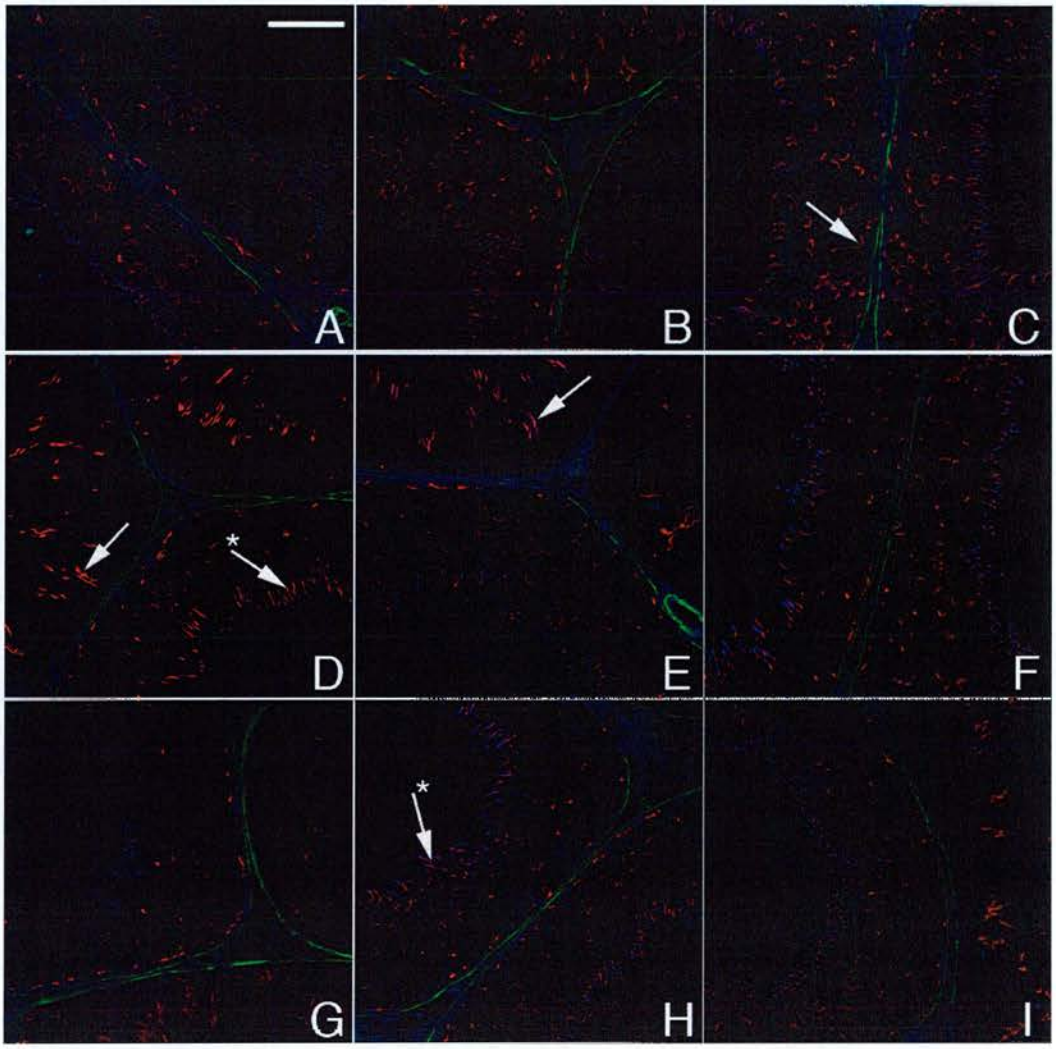


Figure 5-14: Fluorescent immunohistochemistry for expression connexin43 and SMA in testes of adult rats treated with EDS or EDS plus TE. Testes of rats injected with vehicle (panels A-C) and rats treated for 6 days with EDS (panels D-F) or EDS plus TE (panels G-I) were stained using antibodies specific for connexin43 (red) and SMA (green). Staining in the apical compartment is indicated by starred arrows, expression at basal locations is indicated by non-starred arrows. All sections were counterstained with DAPI nuclear stain (blue) and images taken at 40x magnification, the scale bar represents 50 μm.

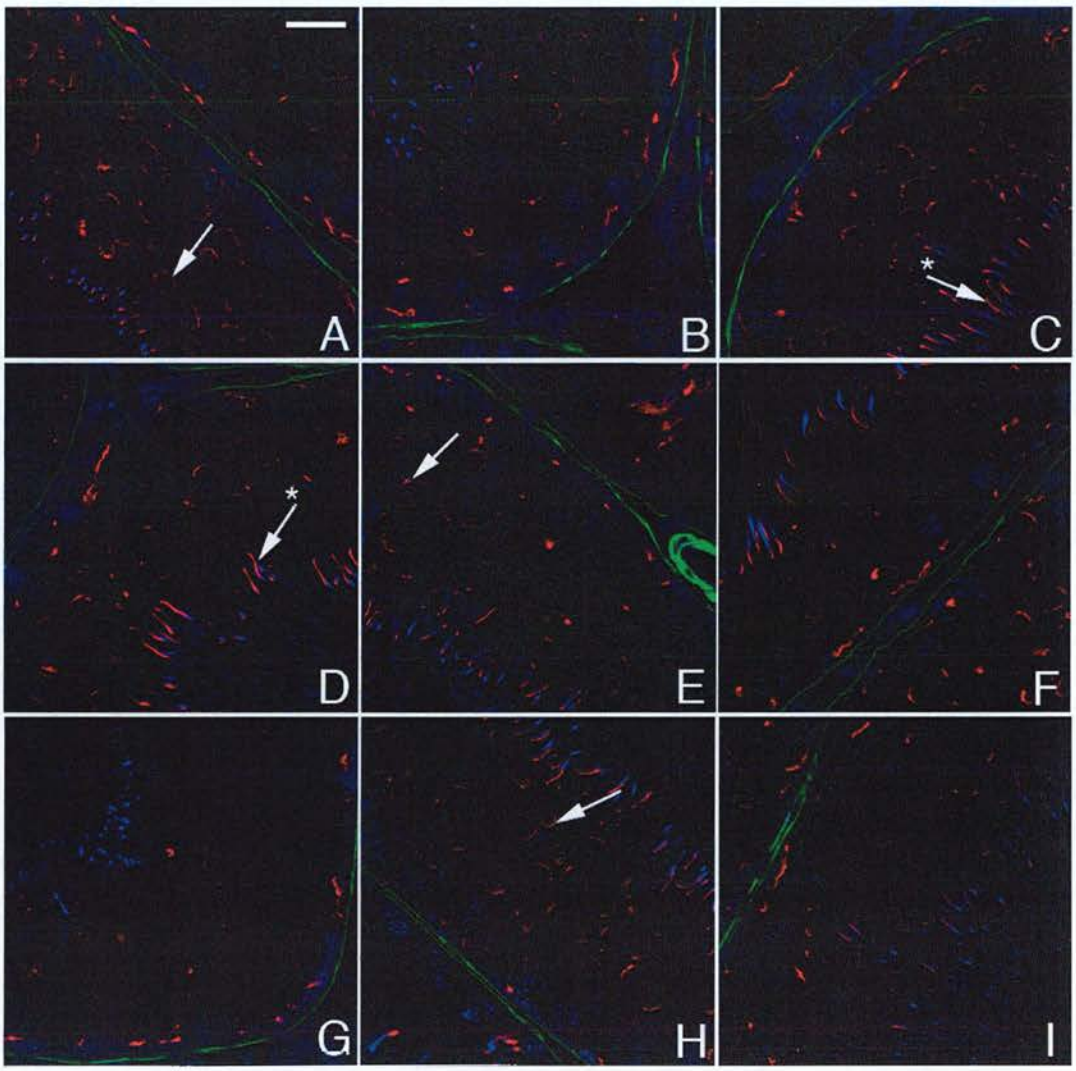


Figure 5-15: Fluorescent immunohistochemistry for expression connexin43 and SMA in testes of adult rats treated with EDS or EDS plus TE. Testes of rats injected with vehicle (panels A-C) and rats treated for 6 days with EDS (panels D-F) or EDS and TE (panels G-I) were stained using antibodies specific for connexin43 (red) and SMA (green). Staining in the apical compartment is indicated by starred arrows, expression at basal locations is indicated by non-starred arrows. All sections were counterstained with DAPI nuclear stain (blue) and images taken at 80x magnification, the scale bar represents 20 μm.

5.4 Discussion

5.4.1

Previous studies have shown that acute androgen withdrawal due to LC ablation by EDS-treatment has a negative impact on GC survival and SC function (Bartlett et al., 1986; Kerr et al., 1993a; McKinnell and Sharpe, 1995; Sharpe et al., 1994; Sharpe et al., 1992). In addition, EDS-treatment reduced seminiferous tubule fluid 6 days after LC ablation and reduced luminal diameters of tubules at stages VI-VIII (Sharpe et al., 1994). Previous studies in isolated seminiferous tubules revealed that the amount of seminiferous tubule fluid secreted was stage dependent whilst greater secretion was demonstrated at stages VI-VIII than during other stages the difference was absent following EDS-treatment (Sharpe et al., 1992). Androgens, and EDS treatment, did not affect synthesis of the secreted proteins, instead they altered the secretory pathway itself (McKinnell and Sharpe, 1995). Since these studies in EDS rats were undertaken, putative targets for androgen regulation have been identified in investigations including array studies on mice with manipulated AR expression (Denolet et al., 2006a; Eacker et al., 2007; Meng et al., 2005) and in mice treated with exogenous steroids (Sadate-Ngatchou et al., 2004). After the problems encountered with isolated SC (chapter 3) and the *in vivo* adenoviral approach (chapter 4), the well established EDS model was used to evaluate expression of putative androgen responsive genes.

The first gene to be evaluated was *AR*, PCR and Western analysis of *AR* expression showed dramatic reductions in the amounts of mRNA and total protein in testes of EDS-treated animals compared to vehicle-treated (control) animals. The reduction in expression of both was reversed in EDS plus TE treated animals. Analysis of the Western blot revealed a significant difference in expression between EDS and EDS plus TE treated animals. The reduction in *AR* mRNA and protein expression in EDS animals may partially be explained by loss of AR-positive LC. However expression was rescued by TE-treatment consistent with androgen-dependent expression in the surviving testicular cell types (SC and PTM). Surprisingly immunohistochemistry on testes of

EDS-treated animals revealed persistence of AR expression in SC and PTM cells. This is in contrast to results in previous studies where both these cell types lost AR expression within 6 days of EDS-treatment (Atanassova et al., 2006; Bremner et al., 1994). The large decline in AR protein detected in the Western blot suggests that AR expression in the SC and PTM was reduced, but the decline in AR expression may have been masked in immunohistochemical studies by high non-specific background staining. High background with positive staining in the cytoplasm of elongated spermatids is not present when other antibodies against AR are used (Bremner et al., 1994) and may be prevented by using one of these other antibodies or by using lower concentrations of the current antibody. Sodium citrate antigen retrieval was used here and by Bremner at al., so should not be the explanation for the high background in these sections.

Expression of *Rhox5* mRNA was androgen-responsive with highest expression in the testes of control and EDS plus TE treated animals, and a dramatic (non-significant) reduction in EDS-treated animals. Total expression of Rhox5 protein did not mirror its mRNA; EDS-treatment caused a 20% reduction compared to testes of control animals, but rather than TE-treatment increasing Rhox5 expression it was associated with a further 20% decrease in protein. *Rhox5* has both distal and proximal promoters (section 1.4.4), expression of *Rhox5* in the testes and epididymis of rats and mice is expressed under the regulation of the proximal promoter (Maiti et al., 1996; Sutton et al., 1998). Despite expression in testis and epididymis being regulated by the same promoter in both mice and rats their patterns of *Rhox5* expression are different. Levels of *Rhox5* transcripts in mice are 10-times greater in testes than in epididymis, but in rats epididymal *Rhox5* expression is more than 10 fold greater than in testes (Sutton et al., 1998). Consistent with the mRNA expression presented above, the androgen-dependent nature of *Rhox5* expression has been described in previous studies. RNase protection assays on testes of mice and rat revealed that expression of transcripts from the proximal promoter, but not the distal promoter, are androgen-dependent being lost in EDS-treated rats and hypophysectomised mice, and retained in animals treated to replenish testosterone levels (Sutton et al., 1998). Indeed an androgen-responsive domain

consisting of two androgen-response elements was identified in the proximal promoter region of the murine *Rhox5* gene (Barbulescu et al., 2001). It was the second response element (ARE-2) linked to *luciferase* that was used in chapter 3 to quantify AR-dependent activation in SK11 cells by androgens. *Rhox5* is expressed in adult mouse SC in a stage-specific manner at stages VII and VIII that parallels AR expression (Lindsey and Wilkinson, 1996b; Sutton et al., 1998) and indicates androgen-responsiveness. Androgen sensitivity of *Rhox5* mRNA expression was also demonstrated in SCARKO mice lacking AR expression specifically in SC, in these animals *Rhox5* mRNA and protein expression were reduced dramatically (De Gendt et al., 2004; Tan et al., 2005). It is not clear why the pattern of androgen responsive expression observed for *Rhox5* mRNA has not been repeated for protein expression, although it is plausible that the Rhox5 protein is highly stable compared to the mRNA and has not brokendown at this relatively early time point. *In vitro* studies on isolated primary mouse SC showed loss of Rhox5 protein even with addition of testosterone to culture media, which suggests androgens alone are insufficient to maintain Rhox5 expression (Sutton et al., 1998). Studies on MSC1 immortalised SC, transiently transfected with *AR* cDNA, demonstrated 5-7 fold increased expression of *Rhox5*, plus 4 other *Rhox* genes, in response to testosterone treatment (Maclean et al., 2005), this result is in contrast to the response in primary mouse SC but in-line with the results presented above.

β3-tubulin mRNA was expressed in a similar pattern to *AR* and *Rhox5*. In testes of rats treated with EDS, expression of *β3-tubulin* was almost completely abolished compared to expression in control animals and could be restored to a level greater than in control testes by treatment with EDS plus TE. The pattern of *β3-tubulin* expression indicates that it is androgen responsive and is in agreement with a previous array study in which *β3-tubulin* was identified as a potential androgen-responsive gene due to its differential expression in SCARKO and wild-type mice (Denolet et al., 2006b). β3-tubulin protein quantified by Western blot did not adopt a similar expression pattern to its mRNA, instead expression in testes of control and EDS-treated rats were approximately equal and expression following EDS plus TE treatment was reduced compared to both other

groups. When a pan β-tubulin antibody was used expression in all 3 treatments was approximately equal. The lack of statistical significance between groups suggests the differences in expression are the result of high variation in expression independent of treatment.

The intimate interactions between SC and GC via junctional complexes, and the extensive evidence linking androgen action via SC in supporting GC maturation have focused attention on proteins that form part of testicular junctional complexes as potential targets for control by androgens. Reports by Braun and co-workers of regulation of claudin3 expression, a component of the BTB, by androgens (Meng et al., 2005) and the appearance of intra-epithelial vacuoles in EDS-treated testes (Bartlett et al., 1986; Kerr et al., 1993b) support the androgen-dependence of junctional protein expression. Expression of *espin* mRNA altered in treated testes with expression in testes treated with EDS plus TE ~50% higher than in control samples, and expression in EDS exposed testes reduced to approximately 10% of control. *Espin* expression has previously been reported as a putative androgen responsive SC product following quantification of gene expression in SCARKO mice (Abel et al., 2008). As described below, immunohistochemistry for espin does not support androgen-responsiveness of espin expression in line with expression of espin in the testes quantified by Western blot, which was largely unaltered by androgen depletion by EDS, or replacement by TE.

The expression and distribution of the junctional proteins espin, connexin43, N-cadherin and ZO-1 were visualised by immunohistochemistry in testis sections collected from animals treated with EDS, EDS plus TE, or vehicle control. In line with the Western blot result, and previous studies that indicate that espin expression at the basal and adluminal ES is not dependent on androgen signalling (Beardsley and O'Donnell, 2003; O'Donnell et al., 2000), expression of espin at the ES was not altered by androgen depletion induced by EDS-treatment, nor did exogenous testosterone treatment alter expression either. Intensity and localisation of ZO-1, N-cadherin and Cx43 were not altered by depletion of testosterone and were not over expressed in response to TE-

treatment. Lack of change to protein expression of ZO-1 and N-cadherin are in contrast to results presented by Xia et al, who reported increased expression of both proteins following androgen depletion in rat testes (Xia et al., 2005). The study also identified diffusion of N-cadherin away from the BTB after testosterone depletion (Xia et al., 2005), which was not observed in the present report. The changes in N-cadherin and ZO-1 expression may not have been sufficiently large in this study to be identified through immunohistochemistry, Xia et al also used Western blotting to identify the increased expression however the change was apparent through immunohistochemistry as well. Altered expression of connexin43 in response to testosterone has not been reported in previous studies, although stage-specific Cx43 expression with greatest expression during stages VI-VIII in rats and mice mimics the expression of AR (Risley et al., 1992). The lack of change in Cx43 expression in response to androgen-depletion and restoration described in the present study is therefore in common with the available literature. Although not demonstrated to be androgen-regulated, expression of Cx43 in SC has been shown to be essential for initiation of spermatogenesis and for proliferation of spermatogonia, SC-specific knock out of Cx43 expression in mice results in infertility and cannot be compensated for by other connexins (Brehm et al., 2007).

The final protein whose localisation and expression was determined through immunohistochemistry was Sdmg-1, the protein is a marker of SC cytoplasm and has been implicated in protein secretion pathways in fetal testes (Best et al., 2008). In testes of control animals expression forms narrow bands of intense staining running from the basement of the tubule inwards towards the lumen of the tubules. The pattern and strong staining was maintained in the testes of animals treated with EDS or EDS plus TE. A lack of changes to expression pattern of Sdmg-1 indicates that depletion of androgens does not impact the distribution of SC cytoplasm and therefore subsequent failure of spermatogenesis is not a consequence of SC losing contact with associated GC due to disruption to its cytoplasm. The staining also revealed no evidence of vacuole formation in the SC of testes treated with EDS or EDS plus TE, early studies reported the occurrence of such vacuoles 7 days after treatment (Bartlett et al., 1986).

Changes in mRNA expression for *AR*, *Rhox5*, *β3-tubulin* and *espin* between treatments were not statistically significant principally because of the small numbers of animals in each of the three treatment groups, in particular the control group which was represented by only two or three animals in each real-time PCR analysis. The four animals studied in the EDS and EDS plus T groups may also have been insufficient. To increase the probability of identifying a statistically significance change, larger numbers of animals, at least five, should be analysed in each of the three groups. Large variation in the expression levels detected between the animals in each treatment group used during the study, in combination with the inadequate numbers of animals used, also impeded statistical significance of the result. High or low levels of mRNA expression were consistently found for all four transcripts in each animal, indicating that each animal exhibited either high or low expression of its transcripts in general. The protein samples, extracted from the same testes as the mRNA, exhibit a much smaller variation in expression, which meant that an observed change in AR protein expression was statistically significant. The changes in mRNA expression detected by Q-RT-PCR were often dramatic, and had it not been for the large inter-animal variation the results may well have been significant. Larger numbers of animals in each treatment group may have overcome these problems. A drawback of the EDS model when investigated through Western blotting and PCR is the inability to differentiate between changes in the different cells that compose the testes, and to compensate for the loss of the LC. In this study we sought to identify changes to gene expression that occur only in the androgen-responsive tubules of the testis, these stage VI-VIII tubules and their proteins make up only a small proportion of the whole testis. Changes in protein expression investigated in these studies may have been insufficient to be detected over the background of proteins expressed in tubules at other stages and in the rest of the testis.

5.4.2 Conclusions

Treatment of rats with EDS, to deplete LC and eliminate testosterone from their testes, appears to have been successful in this study on the basis that LCs were absent in testicular sections and expression of *AR*, *Rhox5*, *β3-tubulin* and *espin* mRNAs, and AR protein were altered as expected in response to androgen depletion and replacement. Androgen-responsiveness of *espin* and *β3-tubulin* mRNA had previously been reported through arrays and Q-RT-PCR on SCARKO and anti-androgen treated mice, so evidence of the same manner of response in a model of acute androgen withdrawal strengthens the evidence for androgen-sensitivity. Despite demonstrating that expression of *Rhox5*, *espin*, and *β3-tubulin* mRNA are androgen-responsive, expression of these proteins assessed by Western blotting does not show an androgen-responsive pattern. The contradiction between reduced mRNA expression and preservation of protein expression in testes following androgen-depletion could be explained if the proteins have long half-lives. A protein with a long half-live could be maintained with only limited degradation for 6 days, after which the testes were collected, despite a dramatic decline in mRNA transcription and translation of new protein.

Results obtained in these studies do not indicate any impact of androgen signalling on the expression or distribution of N-cadherin, ZO-1 or Cx43 proteins within seminiferous tubules. Along with the results for espin protein, these 4 proteins represent components from ectoplasmic specialisations (espin and N-cadherin), gap junctions (Cx43), tight junctions (ZO-1), and desmosome-like junctions (N-cadherin). The lack of consistent decline in expression of junctional proteins does not indicate disruption to SC-GC interaction in response to androgen depletion despite previous reports of GC sloughing in tubules of testosterone suppressed rat (Cameron et al., 1993; O'Donnell et al., 2000) and loss of claudin3 expression at tight junction in testes with SC-specific knockout of AR (Meng et al., 2005). Expression of Sdmg-1 was also maintained following androgen depletion indicating that irrespective of the method by which androgens support spermatogenesis via the SC, the impact of androgen depletion is not by disrupting the distribution of the SC cytoplasm. Sdmg-1's involvement in protein secretion pathways

in fetal testes might have predicted that its expression would be altered in response to changing testosterone levels in adult testes in light of the reduced protein secretion observed by Sharpe and co-workers following androgen-depletion (McKinnell and Sharpe, 1995; Sharpe et al., 1994; Sharpe et al., 1992)

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6 General Discussion

6.1 Introduction

Spermatogenesis is dependent upon functional Sertoli cells to support the GCs with which they interact. Expression of a functional AR in SC is required for complete spermatogenesis (Chang et al., 2004; De Gendt et al., 2004; Holdcraft and Braun, 2004). De Gendt et al. developed a mouse model in which expression of AR was knocked out throughout the body (ARKO) or specifically in SC (SCARKO). Male ARKO mice have a female phenotype and undescended, abnormally small testes located in the abdomen or inguinal region (De Gendt et al., 2004). Spermatogenesis is disrupted in ARKO mice due to failure to support spermatogonial and meiotic GC maturation (Tan et al., 2005). In SC-specific AR knockout mice generated by De Gendt et al. and Chang et al. testes descend normally into the scrotum, however spermatogenesis was disrupted by failures in meiosis during stages VI – XII, resulting in reduced numbers of round and elongate spermatids (De Gendt et al., 2004). Spermatogenesis in a third SC-specific AR knockout model, generated by Holdcraft et al, was also disrupted (Holdcraft and Braun, 2004). During the generation of the AR floxed line used by Holdcraft et al. a hypomorphic mutant was generated with reduced AR expression in SC and other cells. In the mice with SC-specific AR depeltion generated using this line the final stages of spermatid development and release of elongate spermatids from the seminiferous epithelium were disturbed (Holdcraft and Braun, 2004).

Tubules at stages VII and VIII in mice and rats have been identified as androgen responsive (Sharpe, 1994) and SC in these tubules exhibit the most intense immunoexpression of AR protein (Bremner et al., 1994; Suarez-Quian et al., 1996; Zhou et al., 2002). Stage-dependent expression of AR has also been documented in human testes (Suarez-Quian et al., 1999). Several mouse models with exon-specific depletions in *ERα* (ERαKO) and *ERβ* (ERβKO) has been successfully described (Dupont et al., 2000; Krege et al., 1998; Lubahn et al., 1993). A new mouse line with a ubiquitous

deletion of ERβ throughout their bodies are infertile although gross testicular morphology appears normal (Antal et al., 2008). *In vivo* models designed to study impact of steroids on SC, such as ARKO mice, often compromise steroid action during fetal or prepubertal development which potentially disturbs gonadal development. *In vitro* methods allow easier manipulation of steroid hormone action, but lack the complex interactions between SC and associated GCs and PTM cells present *in vivo*. In the present study three approaches were used to investigate the impact of steroid signalling on SC gene expression. First an *in vitro* model (the SK11 cell) was assessed, and gene expression in these cells was quantified with the aim of identifying responsive genes implicated in control of spermatogenesis. Second methods for specific infection of mouse SC within the seminiferous tubule using adenoviral vectors were explored; we intended to use this method to introduce RNAi constructs to knockout AR or ERβ expression specifically in SC. Finally the EDS-treated rat model was used to identify putative androgen responsive genes and validate changes in expression of genes identified as androgen-responsive in previous studies conducted using the SCARKO mouse model.

6.2 Gene expression using an immortalised Sertoli cell line

SK11 cells are immortalised SC derived from testes of day 10 mice whose proliferation and phenotype is sensitive to culture temperature (Walther et al., 1996) due to the presence of a temperature sensitive large T-antigen expressed in the mice from which they were derived (Jat et al., 1991). Previous investigations of gene expression in SK11 cells have demonstrated differential expression of *SGP-2*, *transferrin*, and *Steel factor* between cells cultured in permissive and non-permissive conditions (Sneddon et al., 2005; Walther et al., 1996; Walther et al., 1997).

In line with these previous studies, SK11 cells incubated at permissive (34°C) and non-permissive temperatures (39°C) in this study have different phenotypes: the cells become flattened and demonstrate a gene expression profile that is more similar to mature SC at non-permissive temperatures than under permissive conditions. Expression of *SGP-2*

and *Rhox5* mRNA and protein is increased at non-permissive temperatures and demonstrates a shift from an immature state where expression of *Rhox5* and *SGP-2* is low, to a mature state when expression would be expected to be higher. *In vivo* expression of *Rhox5* gene in mouse, detected by RNase protection assay, increased on day 9 post-partum (Lindsey and Wilkinson, 1996b). The expression of SGP-2 protein is detected in mouse SC from 12 days post-partum and intensity of staining increases between 12 and 50 days post-partum (Tan et al., 2005). The changes in gene expression in cells cultured in non-permissive conditions correspond well with gene expression in day 10 mouse SC, which was the age of the mice from which the cells were derived (Jat et al., 1991). Shift of phenotype from 'immature' to 'mature' following transfer from 34°C to 39°C provides an attractive model for detailed investigation of the mechanism of SC development, or to validate previous studies. The expression of Sdmg-1 at both temperatures might be used as a marker of seminiferous tubule fluid secretion by SC at immature and mature stages of development and the similarity between the amount of total protein at the two temperatures suggest gross secretory function is similar in mature and immature SC. Further investigation of this and other secretory proteins, such as cyclic protein -2, may reveal further details of SC fluid secretions.

Expression of *AR* and *ERβ* mRNA and protein had been previously demonstrated in SK11 cells, and steroid responsiveness had also been demonstrated by quantifying the amount of a luciferase reporter gene produced under control of androgen or oestrogen response elements in the presence of androgen and oestrogen ligands (Sneddon et al., 2005). Therefore we sought to investigate expression of putative steroid-responsive genes and identify novel targets. Unfortunately the SK11 cells used in the current study lacked significant androgen receptor protein and mRNA and this prevented analysis of androgen responsive gene expression in their native state. Successful restoration of *AR* mRNA and its translation into a functional AR protein was achieved by transient transfection with a plasmid containing a full length *AR* cDNA. Expression of an exogenous *Rhox5* promoter-*luc* construct was successfully stimulated by androgen treatment in the *AR*-transfected SK11 cells, however expression levels of endogenous

genes including *Rhox5* that had already been identified by array analysis as up regulated by androgens in SC *in vivo* were not stimulated under the same conditions. Oestrogen receptor β (*ERβ*) mRNA expression and response to oestrogens has been demonstrated in SK11 cells without the need for transfection with an oestrogen receptor construct; as no oestrogen responsive genes was successfully identified in SC validation of the response was limited to stimulation of an exogenous *3xERE-luc* construct. Stimulation of endogenous oestrogen responsive genes, if any are identified, may also be deficient in SK11 cells in the same manner and for the same reasons as androgen-responsive gene expression but this merits investigation in the future. If the SK11 cell line, or any other SC-line, is to be used in the future to study response to AR-dependent gene activation then the androgen response mechanism must be fully functional, and the reasons for the deficiency in stimulation through endogenous promoters should be identified. The function of SC is recognised to be strongly dependent on its testicular niche including interaction with GCs and PTMs (Maguire et al., 1997; McKinnell and Sharpe, 1997; Pineau et al., 1990; Skinner and Fritz, 1985a; Skinner and Fritz, 1985b; Syed and Hecht, 1997; Tirado et al., 2003), and therefore functions including the response to steroid stimulation could be compromised in the absence of factors/interactions with GC and PTM cells. If GCs or PTM cells, or both, are required for full function of SC, modified methods for investigation of SC response to steroids and other functions will be required. Alternative methods for the study of SC function include co-cultures of SC and GC such as described by Syed and Hecht (Syed and Hecht, 1997), culture of isolated seminiferous tubules (McKinnell and Sharpe, 1995) or *ex vivo* cultures of explanted testes (Schlatt et al., 1999). Any of these could be employed if isolated SC cultures prove insufficient for study of steroid signalling on SC.

Notably, SC cultured in the absence of GC cannot undergo cyclic changes characteristic of SC in tubules at different stages of spermatogenesis. The cultured SC will never exhibit stage-specific changes in expression of genes such as: *AR*, *SGP-2*, *cyclic protein 2*, and *connexin43* (Bremner et al., 1994; Maguire et al., 1993; Risley et al., 1992; Tan et al., 2005) all of which have been reported to be expressed to varying levels in tubules at

different stages of the spermatogenic cycle. SK11 cells in culture have a flattened appearance in contrast to the cell morphology observed *in vivo* in seminiferous tubules of adult testes, further evidence of variation between *in vitro* and *in vivo* SCs.

The greatest success in identifying androgen responsive genes in SC has been achieved using arrays to compare expression between control testes and those with SC-specific ablation of AR expression (Abel et al., 2008; Denolet et al., 2006a) or after treatment with anti-androgens (e.g. Flutamide) (Denolet et al., 2006a). To identify putative oestrogen responsive genes in SC a similar system using a floxed *ERβ* allele (Antal et al., 2008) and the *AMH-Cre* mouse line could be used to establish a SC-specific ERβ knockout. Expression in the testes of this model could be compared to that in control testes via array analysis to identify putative targets for investigation of oestrogen-response in SC.

6.3 Gene expression in Sertoli cells using adenoviral constructs

Adenoviral vectors have been used in previous studies to specifically introduce transgenes into SC by delivering the virus into the lumen of seminiferous tubules via one of three routes, 1) direct injection into the tubule lumen, 2) injection into the rete testis, or 3) canulation of an efferent ductule (Blanchard and Boekelheide, 1997; Kanatsu-Shinohara et al., 2002; Scobey et al., 2001). These studies successfully induced efficient SC-specific expression of a phosphorylation deficient mutant of CREB, LacZ or Steel factor *in vivo* and *in vitro*, without expression in associated GCs (Blanchard and Boekelheide, 1997; Kanatsu-Shinohara et al., 2002; Scobey et al., 2001). The *Rhox5* promoter has been used *in vivo* to knockdown WT-1 expression by driving specific expression of RNAi in SCs of transgenic mice (Rao et al., 2006). We carried out a pilot study to investigate whether an adenoviral vector could be delivered into seminiferous tubules by injection into an efferent ductule to infected only SC without damaging the tubules. The intention was to harness the siRNA technology by delivery directly, and acutely, into the testes of adult mice.

When adenoviral vectors were injected into the testicular tubule lumens, infection was SC specific. Previous studies *in vivo* and *in vitro* have also reported that GC are not infected by adenoviral vectors (Blanchard and Boekelheide, 1997; Scobey et al., 2001). The specificity of infection cannot be explained by the distribution of CAR, because it is expressed by both SC and GC at junctions between these cell-types (Wang et al., 2007), and therefore should provide access for the virus into both cell-types. Expression of CAR at intercellular junctions in columnar epithelia makes the receptor inaccessible to the virus, similar restrictions may explain adenoviruses' failure to infect GCs but still does not explain specific infection of SCs that are adjacent to the GCs (Pickles et al., 1998; Wang et al., 2007).

Adenoviral vectors enter target cells by attachment to the CAR receptor via fibre knobs attached to the virus' capsid (Henry et al., 1994). Attachment of viral particles to CAR protein competes with CAR-CAR interactions within the tight-junctions between adjacent cells which disrupts the stability of these junctions (Walters et al., 2002; Wang et al., 2007). The junctions are components of the blood-testis barrier that exists between adjacent SCs. In addition to direct interference with junctions by competing for binding with components of the complexes, binding of adenovirus to CAR may induce signalling cascades involving Rho GTPase, Rac-1, or Cdc42 which open occludin- and claudin-based tight junction complexes (Wang et al., 2007). A key finding of the current work, presented in chapter 4, was the development of intraepithelial vacuoles and loss of GCs resulting from adenoviral infection. Disturbance of junctional complexes due to infection with the adenoviral vector may be one explanation for the appearance of those vacuoles. Future work aimed at addressing this possibility could include immunohistochemistry for proteins previously localised to tight junctions such as ZO-1, claudin-3 and -11, and occludins. This would allow us to determine whether their expression is altered or more probably whether their distribution changes following adenoviral infection in line with loss of interaction between tight junction proteins in opposing SC cells. Wang and Cheng (2007) discussed the impact of adenoviral

interaction with CAR found in tight junctions within the blood-testis barrier, but other junctions exist within the seminiferous tubules which could be targets of the signalling cascades induced by adenovirus infection. Although the expression level of espin, a component of ectoplasmic specialisations, was not altered its distribution was disturbed and this could be one of many junctional protein targets of the signalling cascade.

An alternative explanation for the disruption of seminiferous tubule structure in response to adenoviral infection is that introduction of the virus stimulated an inflammatory response. Increased expression of inflammatory cytokines, interleukin-1α and -6, has been reported in isolated SCs in response to incubation with lipopolysaccharide or residual bodies in culture (Syed et al., 1993; Syed et al., 1995). As a preliminary experiment to investigate whether SC might respond to adenoviral infection by initiating a proinflammatory response, expression of *IL-6* mRNA and protein were quantified in SK11 cells incubated with a dose of adenovirus previously identified as cytotoxic. Increased expression of IL-6 was not detected in response to infection of SK11 cells at a high MOI, although the amount of IL-6 synthesised in the cells grown at the non-permissive temperature was increased compared to proliferative cells. Future work could determine whether failure to express increased IL-6 in response to viral infection was due to a failure of SC to respond with increased expression of IL-1α which stimulates IL-6 expression via autocrine signalling. Although it appears unlikely that SK11 cells can be used to investigate the interleukin-mediated response of SC to adenoviral infection, if the *in vivo* infections of testes with the virus were repeated testes could be preserved differently to allow mRNA extraction and quantitative analysis by Q-RT-PCR. Analysis of IL-1α and -6 protein expression could also be assessed in isolated tubules cultured in the presence of adenovirus to allow quantification of interleukins in the seminiferous tubule fluid. Isolated seminiferous tubules would be used to maximise the probability of detecting changes in interleukin expression as levels would be expected to be low even following infection and analysis in whole testes or quantification in blood stream would diminish sensitivity. Detection of interleukin expression could be related to events such as recruitment of macrophages and

neutrophils in response to adenoviral infection which was observed in some of the more severely disrupted testes resulting from injection of the highest concentrations of virus. The secretion of IL-1α and -6 would be compared to the expression of these interleukins in response to inflammation induced with LPS and residual bodies in previous studies (Syed et al., 1993; Syed et al., 1995).

In addition, some of the damage associated with intra-testicular injection of adenoviral vectors could be a consequence of the injection process itself or one of the other components (saline solution or trypan blue) of the injection vehicle. The severity of the damage sustained by the tubules correlates with the quantity of virus injected into the testis, with greater disruption associated with larger quantities, which suggests that the presence of the virus is at least partially responsible for the effects. The volume injected into each testis was small, 50µl, in comparison to the 100 – 150 µl injected via the same route by Ogawa et al., although they did report ischemia and complications to GC transplantation as a consequence of 'excessive internal testicular pressure' caused by efferent ductule injection (Ogawa et al., 1997). Despite this the same volume of vehicle was injected at each dosage used during the study so cannot explain the variation in damage witnessed. The volumes and sites of injection used in previous studies are shown in Table 6-1 for comparison, the only other study in mice with a directly comparable injection site was performed by Kanatsu-Shinohara et al. who used much lower volumes of vehicle and only observed occasional inflammation in response to injections. Under appropriate circumstances a short control study could be undertaken in which 50 µl of the saline/trypan blue vehicle would be injected into testes using an identical method to the previous injections. Histology of these tissues would be compared to uninjected and adenovirus infected testes to determine what impact the injection process and components of the vehicle had on the tubule structure.

Table 6-1: Comparison of sites of injection and volume of solution injected

Paper	Species	Site of Injection	Volume injected (µl)
(Ogawa et al., 1997)	Mouse	Seminiferous tubule	400 - 500
		Efferent ductule	100 – 150
		Rete testis	100 - 150
(Blanchard and Boekelheide, 1997)	Rat	Rete testis	5 - 10
		Interstitial space	75
(Scobey et al., 2001)	Rat	Rete testis or Seminiferous tubule lumen	50
(Kanatsu-Shinohara et al., 2002)	Mouse	Efferent ductule	3 or 10
Present study	Mouse	Efferent ductule	50

Stage specific loss of pachytene spermatocytes and retention of other GCs at four days after infection suggests a particular sensitivity of zygotene spermatocytes in stage XI-XII tubules to disruption by viral infection at the time of injection. It would be valuable to determine whether features specific to zygotene spermatoycytes or SC at stages XI-XII are altered in response to viral infection. Although deficiencies in the expression of Cx43 and distribution of espin have been identified in association with infection additional tubular deficiencies are possible. Factors secreted by SC that affect GC development such as activin, steel factor and GDNF (Marziali et al., 1993; Matsui et al., 1991; Meehan et al., 2000; Meng et al., 2000) would be prime candidates for quantification in SC at stages XI-XII and other stages following adenoviral infection. Developing a method in which stage-specific secretion could be assessed whilst maintaining the GC complement and testicular niche would be a challenge.

If use of SC-GC co-cultures became necessary to improve *in vitro* studies of SC function, the capacity of adenoviral infection to infect SC exclusively could be used to specifically modify SC function in the co-cultures.

6.4 Gene expression in EDS-treated rats

Although it has not been possible to confirm androgen responsive expression of *AR*, *Rhox5*, *β3-tubulin* or *espin* mRNA or protein in SK11 cells, androgen-dependent expression of mRNA for all four genes has been demonstrated in EDS-treated rats. Androgen-responsive expression of *Rhox5*, *β3-tubulin* and *espin* mRNA have been detected previously using array analysis of testes from SCARKO mice or testes of flutamide treated mice compared with controls (Abel et al., 2008; Denolet et al., 2006a). Historically the EDS-model has been one of several that have demonstrated the necessity for androgen signalling to maintain normal male fertility. Despite these findings to date few androgen responsive genes have been positively identified in testes or isolated testicular cells (Abel et al., 2008; Bremner et al., 1994; Denolet et al., 2006a; Lindsey and Wilkinson, 1996b; Turner et al., 2001). One common explanation for the limited data relating to androgen responsive genes in SC is the influence of GCs on this cell population. Use of isolated Sertoli cells and many of the *in vivo* methods used to study androgen response in SC depleted some, or all, of the GC complement and would have had an additional impact on SC function (Maddocks et al., 1992; Maguire et al., 1997; McKinnell and Sharpe, 1997; Pineau et al., 1990; Tirado et al., 2003) preventing identification of the androgen-responsive genes. Unless better cell lines can be developed, as described in section 6.2, the best *ex vivo* system available for study of SC function may be testis explants. The EDS rat model is one of very few *in vivo* alternatives to generating transgenic mouse models or treating animals with anti-androgens. A benefit of the EDS models is that it interferes with androgen signalling but retains an almost complete GC complement as long as tissues are collected within 6 days of treatment (Turner et al., 2001). The SC also maintain contact with peritubular myoid cells which also interact with SC to maintain their function (Skinner and Fritz, 1985a; Skinner and Fritz, 1985b). A potential drawback of the EDS model is the

increased level of FSH that develops following EDS treatment (Bartlett et al., 1986; Tena-Sempere et al., 1993). FSH and testosterone have been shown to regulate similar processes within the testis including spermatogenesis (Allan et al., 2004; Kerr et al., 1992), indeed Abel et al identified genes (*Espn*, *Msi1*, and *Slc7a4*) that were synergistically regulated by both FSH and androgen action in SC. The study demonstrates that both FSH and androgen signalling via SC act to maintain meiotic germ cells (Abel et al., 2008). Expression of other genes identified as androgen responsive in SC; including *Rhox5*, *Aqp8*, *Tjp1* and *Gata1*; were not influenced by FSH signalling (Abel et al., 2008). If FSH is able to compensate for the loss of testosterone levels in the EDS animals then putative androgen responsive genes identified in previous array studies may be wrongly discounted based on studies in EDS rats.

Puzzlingly, although expression of *espin* mRNA was reduced dramatically by EDS-treatment, no change in total protein or pattern of protein expression detected by immunohistochemistry was detected. Therefore we cannot rule out the possibility that the lack of detectable changes in Cx43, ZO-1 and N-cadherin protein expression by immunohistochemistry may mask changes in mRNA expression of these genes through Q-RT-PCR, and this requires further investigation. Further genes identified by Denolet et al and Abel et al. in array studies on SCARKO tissue suggest other putative targets for androgen signalling in SC (Abel et al., 2008; Denolet et al., 2006a), and the expression of mRNA for these genes could be assessed using the same EDS-treated rat model. The genes identified in the arrays include serine protease inhibitors, cell adhesion molecules, cytoskeletal components, members of the extracellular matrix, and proteins involved in solute transport (Abel et al., 2008; Denolet et al., 2006a).

Previous studies have reported the impact of androgens on the secretion of proteins that constitute seminiferous tubule fluid (Sharpe et al., 1994; Sharpe et al., 1992). As Sdmg-1 has been reported to be involved in fetal secretory pathways (Best et al., 2008) we investigated this protein as a potential target for androgen action. Secretion of proteins that contribute to seminiferous tubule fluid can either be constitutive or androgen-

regulated. The major secretory proteins SGP-1 and -2 are both constitutively secreted and unaffected by androgen action, in contrast cyclic protein 2 (CP-2) plus other SC and GC proteins, are secreted by regulated pathways in response to androgens (McKinnell and Sharpe, 1995). Proteins expressed through androgen-regulated pathways exhibit stage-specific expression, with increased expression during 'androgen-responsive' stages (McKinnell and Sharpe, 1995). The lack of response to androgen depletion or replacement when expression of Sdmg-1 is investigated could indicate that Sdmg-1, like the constitutively expressed SGP-1 and -2, is not regulated by androgens. Further investigations, including Western blotting, of Sdmg-1 expression pattern in control testes could confirm whether Sdmg-1 adheres to an expression pattern in common with SGP-1 and -2 (constitutive expression) or CP-2 (stage and androgen-dependent). A cursory review of Sdmg-1 immunohistochemical staining suggests that expression is constitutive and therefore unlikely to be androgen responsive.

6.5 Conclusions

The question of whether isolated SC can be sustained in culture in conditions that are sufficiently like those experienced by SC *in vivo* thereby allowing investigation of androgen-responsiveness and other features of SC biology cannot be answered by the results of these studies. Although expression of functional AR and ERβ proteins and stimulation of exogenous reporter constructs in SK11 cells do indicate that this cell has potential, it has the disadvantage of being generated from an immature (10 days post partum) mouse. Further studies with this line of cells are required to determine whether stimulation of endogenous genes by steroid treatment can be achieved by simple modifications such as stable transfection with *AR* and/or AR cofactors.

Array data from *in vivo* models with disruption to androgen signalling has been valuable in identifying numerous putative androgen-responsive genes, whose mRNA expression is raised or lowered by loss of androgen-signalling. Refinement of the use of SK11 cell cultures and the EDS-rat model to resolve the lack of endogenous transcriptional activation in response to AR activation and to overcome issues of statistical significance

with larger samples sizes respectively would allow the SK11 and EDS models to be used to validate the androgen-responsive nature of these genes' expression. The SK11 cells could also play a similar role in validating oestrogen responsive gene expression, should such genes be identified perhaps through arrays in ERβ cell-specific knockout model via a similar scheme as to that used with the cell-selective AR knockouts. The results collected during these studies have validated the androgen responsive expression of *AR*, *Rhox5*, *espin* and *β3-tubulin* indicated by array analysis in previous studies (Abel et al., 2008; Denolet et al., 2006a).

Use of adenoviral vectors for infection of SC *in vivo* without significant disruption to seminiferous tubules is not possible because adenoviral infection results in formation of vacuoles in the seminiferous epithelium and loss of GCs at specific stages of development, and at the highest doses collapse of tubule structure and immune cell invasion. Adenoviral vectors specifically infect SC in seminiferous tubules, and efficiently infect SC *in vitro* resulting in expression of a transgene in the absence of a cytotoxic effect. The adenoviral vector therefore may be useful for specific infection of SC in co-cultures of SC and GC if required for future studies. The results presented in these studies have precluded the use of adenoviral vectors introduced by intratesticular for targeted knockdown of AR, or ERβ, in SC. Use of other viral vectors, such as lentivirus, should be considered in place of adenovirus to introduce RNAi specifically into SC. A previous study compared the use of viral vectors, including both adenovirus and lentivirus, for introduction of transgene specifically into SC. The study concluded that lentiviral vectors were preferable to adenoviral vectors because lentivirus was associated with SC-specific expression over a six-month period without disruption to spermatogenesis, in contast to adenoviruses which caused a significant reduction in testis size (Ikawa et al., 2002). Viral vectors that require dividing cells to infect would not be suitable for these studies because adult SC are differentiated and have ceased proliferation (Sharpe et al., 2003).

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